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**THE
PhD
PROGRAM
DIMET**

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Translational and Molecular
Medicine (DIMET)
is an inter-departmental
project between the School
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PhD

**PROGRAM IN TRANSLATIONAL
AND MOLECULAR MEDICINE**

DIMET

**UNIVERSITY OF MILANO-BICOCCA
SCHOOL OF MEDICINE AND SCHOOL OF SCIENCE**

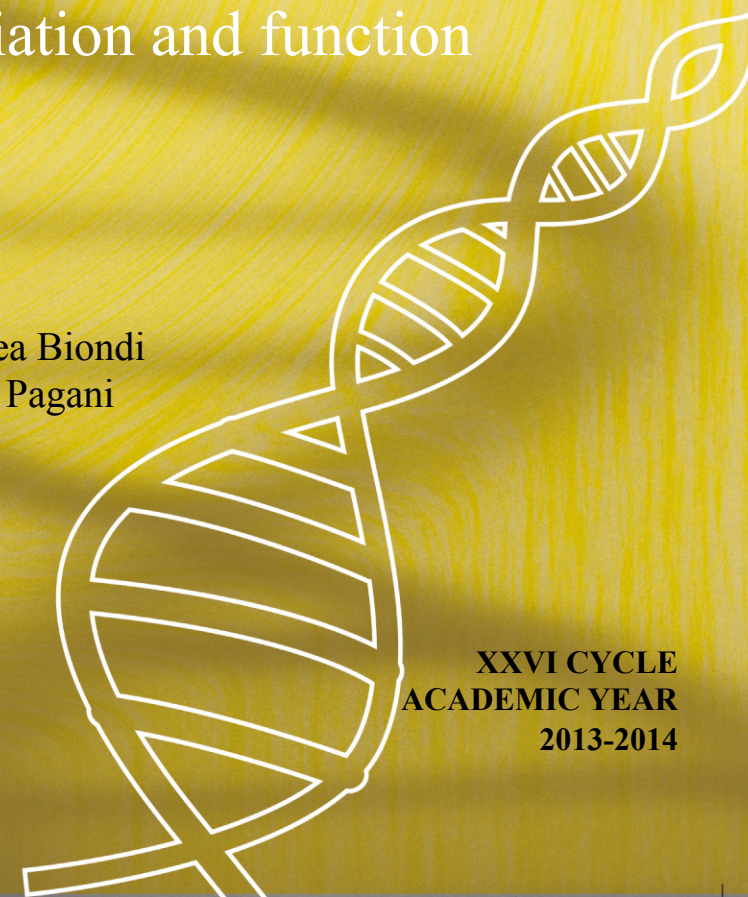
microRNAs role in human follicular helper
T cells differentiation and function

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**XXVI CYCLE
ACADEMIC YEAR
2013-2014**



A Francesca, luce della mia vita

Ad Andrea, per essermi sempre accanto

*Ai miei genitori, per l'amore e il sostegno che mi hanno dato e che
sempre mi daranno*

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1. INTRODUCTION

1.1 Follicular helper T cells (T_{FH})

The induction of protective immunity against invading pathogens depends on the generation of a proper type of the immune response. The cells of innate immune system, such as macrophages, neutrophils and dendritic cells (DCs), provide a first line of defense against many common microorganisms and are essential for the control of common bacterial infections. This first line of defence is immediate and non-specific and allows the protection of our body from pathogens in the first hours of infection and for about 4-7 days, before the initial adaptive immune response starts to be effective. The activation of innate immune response results in the production of cytokines and chemokines that in turns attract and activate neutrophils and monocytes from the bloodstream. This causes the beginning of the process of inflammation that triggers adaptative immune response. In secondary lymphoid organs dendritic cells (DCs), but also macrophages and B cells, function as antigen presenting cells (APCs), that present protein antigen to naive T cells with the major histocompatibility complex (MHC) class I or II molecules. Each naive lymphocyte entering the bloodstream bears antigen T cell receptor (TCR) of a single specificity, that is determined by a unique genetic mechanism that operates during lymphocyte development in the thymus. From the thymus, a primary lymphoid organ where negative selection of autoreactive TCRs of newly formed T cells mainly occurs, emerge two main classes of T cells: naive $CD4^+CD8^-$ T cells

and CD4⁺CD8⁺ T cells. While CD8⁺ T cells bind peptide-MHC class I complexes (pMHCI), exposed on cells infected from viruses or cytoplasmic pathogens and kill the infected cells secreting cytotoxins or expressing FASL to induce target cell apoptosis (cytotoxic T cells), CD4⁺ T cells bind peptide-MHC II complexes (pMHCII) on APCs and produce cytokines that activate other components of the immune system (helper T cells). Specific CD4⁺ T helper cell subsets differentiation depends on the cytokine milieu of the microenvironment and also from concentration antigens, type of APCs, co-stimulatory molecules that in turn promote different patterns of gene expression, including T cell subsets specific transcription factors (Table 1). The generation of effector cells from a naive T cell takes several days. At the end of this period, the CD4⁺ effector T cells subsets leave the lymphoid organs and re-enter the bloodstream to migrate to sites of infection. These effector cells compose the cellular branch of adaptive immunity, providing a highly specific cellular defence against different kinds of pathogens and also creating an memory T cells that can be quickly re-activate on subsequent re-encounter of the same pathogen. Adaptive immune response is made up also by humoral immunity, mediated by antibody secreted by B cells. Secondary lymphoid organs are highly organized structures composed of B (follicles) and T cells zones where, at the interface of these two areas, T helper cells that has been primed can interact with cognate B cells primed with the same T cell antigen: T cells provide important survival and differentiation signals to cognate B cells promoting their differentiation into plasma cells secreting high-affinity antibody or memory B cells, mediating humoral immunity

(Fig. 1). The CD4⁺ T helper cells subset specialized in providing survival and differentiation signals to B cells for their specialization and maturation in secondary lymphoid organs are follicular helper T cells (T_{FH}). (Fig.1)

Table 1: Effector subsets of CD4⁺ T cells: ontogenic and functional requirements, and roles in disease²

CD4 ⁺ T cell subset	Inducing cytokines	Activated STATs	Transcription factors	Suppressing cytokines	Canonical cytokines produced	Roles in host protection	Associated pathologies	Refs
T _H 1 cells	• IL-12 • IFN γ	• STAT4 • STAT1	T-bet	IL-4 and IL-10	IFN γ	• Antiviral and antimicrobial immunity • Cell-mediated immunity	• Mendelian susceptibility to mycobacterial disease (decrease in T _H 1 cells) • Multiple sclerosis (increase in T _H 1 cells)	1,121, 122
T _H 2 cells	• IL-4	• STAT6	GATA3 and MAF	IFN γ	IL-4, IL-5 and IL-13	• Immunity to extracellular parasites	• Allergy, asthma or eczema (increase in T _H 2 cells)	1,123
T _H 17 cells	• IL-23 and IL-1 β • IL-6 and IL-1 β • TGF β	• STAT3	ROR γ t and ROR α	• IL-4, IFN γ , IL-27 and IL-2 • TGF β (suppresses IL-22 expression)	IL-17A, IL-17F, IL-21, IL-22 and IL-26*	• Protection at mucocutaneous sites • Antimicrobial immunity (for example, against <i>Candida</i> spp. and <i>Staphylococcus</i> spp.) • Inflammatory bowel disease	• Inflammatory bowel disease (increase in T _H 17 cells) • Susceptibility to fungal infections (decrease in T _H 17 cells)	1,121, 122,124
T _H 9 cells	• TGF β • IL-4	• STAT6	PU-1 and IRF4	IFN γ and IL-27 [†]	IL-9	• Protection against helminth infections	• Allergy (atopic dermatitis) and asthma (increase in T _H 9 cells)	125
T _H 22 cells	• TNF • IL-6	• STAT1 • STAT3 • STAT5	ROR γ t and AHR	High doses of TGF β	IL-22	• Barrier immunity (skin, intestines and airways) • Enhancement of innate immunity • Tissue regeneration	• Allergy (atopic dermatitis) (increase in T _H 22 cells) • Inflammation at joints and barriers (increase in T _H 22 cells in mice)	126
T _{Reg} cells	• TGF β and IL-2	• STAT5	FOXP3	IL-6	TGF β and IL-10	• Immune suppression	• IPEX syndrome (decrease in T _{Reg} cells)	75
T _{FH} cells	• IL-6, IL-21 and/or IL-27 • IL-12	• STAT3 • STAT4 • STAT1	BCL6, IRF4, MAF and BATF	IL-2 and IL-10	IL-21, IL-4 and IL-10	• Help for B cell activation or differentiation • Generation of long-lived antibody responses	• Humoral immunodeficiency (decrease in T _{FH} cells) • Autoimmunity (increase in T _{FH} cells) • T cell lymphoma (increase in T _{FH} cells) (see TABLE 2)	23–25, 45

AHR, aryl hydrocarbon receptor; BATF, basic leukemia zipper transcriptional factor ATF-like; BCL6, B cell lymphoma 6; FOXP3, forkhead box P3; GATA-3, GATA-binding protein 3; IFN γ , interferon- γ ; IL, interleukin; IPEX, immunodysregulation, polyendocrinopathy and enteropathy X-linked; IRF4, interferon-regulatory factor 4; ROR, retinoid-related orphan receptor; STAT, signal transducer and activator of transcription; TGF β , transforming growth factor- β ; T_{FH}, T follicular helper; T_H, T helper; TNF, tumour necrosis factor; T_{Reg}, T regulatory cells.

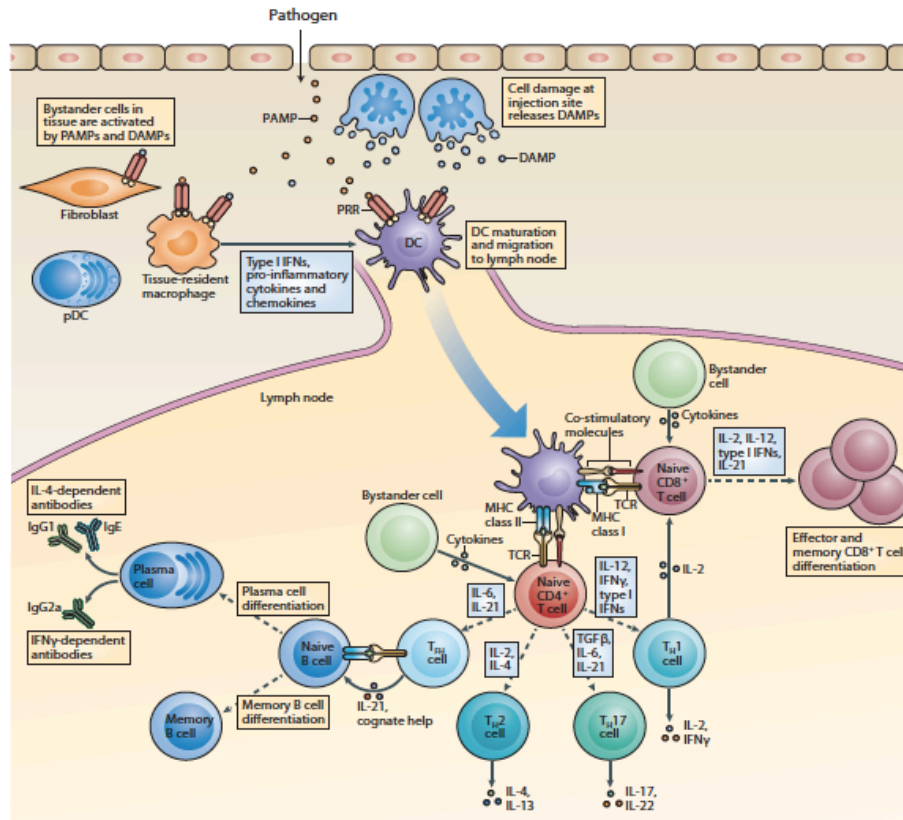


Figure 1: Induction of adaptive immune responses (T helper cell differentiation) through PRR-mediated dendritic cell activation¹.

Pathogens express many pathogen-associated molecular patterns (PAMPs) or may induce the local release of damage-associated molecular patterns (DAMPs). These PAMPs and DAMPs are detected directly by pattern-recognition receptors (PRRs) expressed by DCs, leading to DC activation, maturation and migration to the lymph nodes. In the lymph nodes, the activated DCs may present antigens to T cells, provide them with co-stimulatory signals and stimulate their differentiation by providing a favourable cytokine milieu. Depending on the cytokine milieu, CD4⁺ T cells may differentiate into various T helper (T_H) cell subtypes. T_H cells may also acquire a T follicular helper (T_{FH}) cell phenotype and help in the activation of cognate B cells, thereby promoting the entry of these B cells into the plasma cell pathway or the germinal centre pathway. In addition, the cytokine expression profile

of T_{FH} cells can dictate B cell isotype switching. Depending on the balance between activating cytokines (and most often with the help of T_{HI} cell-derived IL-2), activated $CD8^+$ T cells differentiate into effector and memory $CD8^+$ T cells. pDC, plasmacytoid dendritic cell; TCR, T cell receptor; TGF- β , transforming growth factor- β .

T_{FH} cells in humans were initially described in 2000 and 2001, when several groups reported that a large proportion of $CD4^+$ T cells in tonsils have a unique phenotype, expressing high levels of B cell zone-homing chemokine receptor C-X-C-chemokine receptor 5 (CXCR5), very low levels of the C-C chemokine receptor type 7 (CCR7) and therefore localized to B cell follicles, including germinal centers (GCs)^{3,4}. T_{FH} cells undergo a stepwise differentiation process critically dependent on the expression of transcriptional repressor B-cell lymphoma 6 (BCL6)⁵. GCs are very important histologically distinct structures that arise within B cell zones (follicles) of secondary lymphoid tissues, where the interrelated and multifaceted processes of B cell affinity maturation (somatic hypermutation, SHM, and selection), class switch recombination (CSR), plasma cell differentiation, and memory B cell differentiation predominantly occur^{6,7}. This process ensures the development of long-lived humoral immunity after infection or vaccination with T cell-dependent (TD) protein antigens (AGs) and it's a unique feature of the mammalian immune system. These features make the GC key to protective immunity against many pathogens. T_{FH} cells are essential for the GC formation and are required to provide cell survival and proliferation signals to GC B cells, during the maturation process into high-affinity plasma cells and memory B cells. These B cell types are essential and

important for lasting protection against infections: high-affinity long lived plasma cells then move and reside in bone marrow and secrete high-affinity antibodies for long periods of time, while memory B cells can be rapidly activated upon antigen re-encounter (Fig. 2).

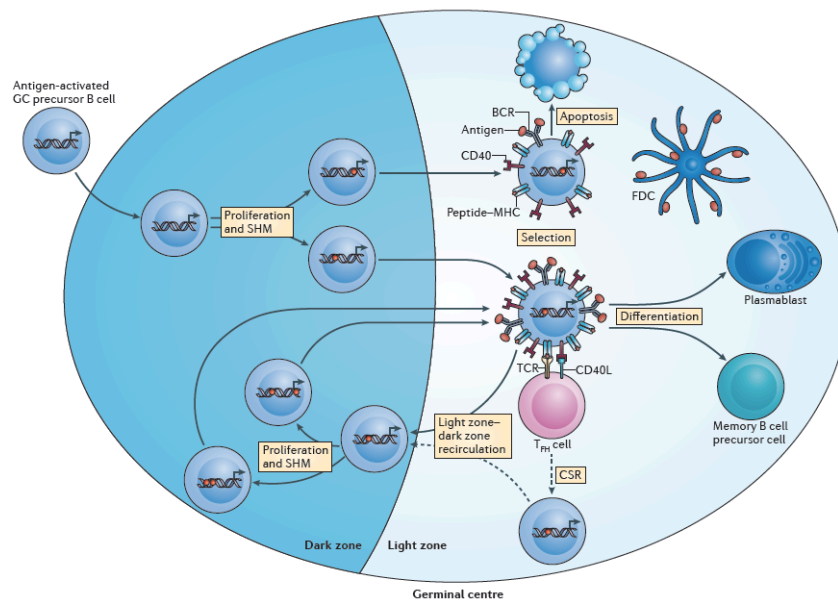


Figure 2: The germinal centre microenvironment⁸. Antigen-activated B cells differentiate into centroblasts that undergo clonal expansion in the dark zone of the germinal centre. During proliferation, the process of somatic hypermutation (SHM) introduces base-pair changes into the V(D)J region of the rearranged genes encoding the immunoglobulin variable region (IgV) of the heavy chain and light chain; some of these base-pair mutations lead to a change in the amino-acid sequence. Centroblasts then differentiate into centrocytes and move to the light zone, where the modified antigen receptor, with help from immune helper cells including T_{FH} cells and follicular dendritic cells (FDCs), is selected for improved binding to the immunizing antigen. Newly generated centrocytes that produce an unfavourable antibody undergo apoptosis and are removed. A subset of centrocytes undergoes immunoglobulin class-switch recombination (CSR). Cycling of

centroblasts and centrocytes between dark and light zones seems to be mediated by a chemokine gradient. Antigen-selected centrocytes eventually differentiate into memory B cells or plasma cells.

T_{FH} cells have been recognized to be a separate subset of CD4⁺ helper T cells, distinct from T_{H1}, T_{H2} and T_{H17} cells and can be distinguished from other CD4⁺ T cell subsets by their low expression levels of cytokines (IFN- γ , IL-4, and IL-17) and transcription factors (T-bet, GATA-3, and ROR γ t) characteristic of T_{H1}, T_{H2}, and T_{H17} cells, respectively⁹⁻¹¹. In addition to express high levels of CXCR5 and low levels of CCR7, T_{FH} cells also downregulate IL-7R α and express a unique combination of effector molecules that are critical for their development and function (Table 2 and Fig. 3).

CD40 ligand (**CD40L**) is the only ligand for CD40 that is constitutive expressed from B cells and it's important for B cell activation, proliferation and survival *in vitro* and *in vivo*. CD40-CD40L engagement is a bidirectional signalling complex: it is critical for the maintenance of GC B cells and also for T cell priming and maintenance in most *in vivo* contests. In the absence of CD40L on T_{FH} cells or CD40 on B cells the block in GC development is absolute¹⁴. The signaling lymphocyte activation molecule (**SLAM**) family of receptors, SLAM (CD150), LY9 (CD229, SLAMF3), 2B4, CD84 (SLAMF5), Ly108 (SLAMF6, NTBA in humans), and CRACC (CS1, CD319, SLAMF7), are self-ligands (with the exception of 2B4, which binds CD48) that are differentially expressed on the surface of T cells and mainly in GC B cells¹⁵. CD150, CD229, CD84 and Ly108 are the members of SLAM family receptors mainly expressed on T_{FH} and GC T_{FH} cells and GC B cells.

SLAM-associated protein (**SAP**), the product of the *SH2D1A* gene, is an SH2-domain adaptor protein that binds to the cytoplasmic tails of SLAM family receptors: SLAM ligation on thymocytes enhances SAP recruitment, SLAM tyrosine phosphorylation, and the recruitment and phosphorylation of SHIP, docking protein 1 (Dok1), Dok2, and Ras-GTPase activating protein (Ras-GAP)¹⁶. SAP regulates intracellular signaling by functioning as an adaptor protein through its ability to bind the SH3 domain of Fyn¹⁷. Consequently, SAP can simultaneously associate with SLAM and Fyn to form a trimolecular complex. SAP is specifically upregulated in GC T_{FH} cells and its expression is essential for GC T_{FH} cell development and the generation of the majority of memory B cells and memory plasma cells¹⁸. In the absence of SAP, T_{FH} cells have defective adhesion to GC B cells and fail to be retained in GCs¹⁹: as a result, insufficient help is provided by SAP-deficient T_{FH} cells to B cells. SAP is essential for GC development also for induction of IL-4 production by T_{FH} cells¹⁴, that has an antiapoptotic activity on B cells.

Inducible T cell-costimulator (**ICOS**) is a CD28-superfamily costimulatory molecule that is upregulated in all CD4⁺ T cells upon activation. It is highly expressed on T_{FH} cells and is important for GC formation because ICOS interaction with ICOS ligand (ICOSL) expressed on DCs and B cells provides signals to CD4⁺ T cells required for initiation and maintenance of T_{FH} cells differentiation^{14, 15}. ICOS recruits phosphatidylinositol-3-kinase (PI3K) and downstream signal via AKT supports IL-21, IL-4 and transcription factor MAF expression by T_{FH} cells^{15, 20}. ICOS has been demonstrated to promote T_{FH} cells differentiation via early induction of the T_{FH} cells

master transcription factor BCL6²¹, while a very recent paper showed in mice shows that ICOS targets another transcriptional repressor Krüppel-like factor 2 (Klf2), a negative regulator of T_{FH} cells maintenance²².

Programmed cell death 1 (**PD-1**) is a potent inhibitory receptor highly expressed on GC T_{FH} cells that binds PD1L1 and PD1L2 ligands expressed on GC B cells. It provides a potent inhibitory signals (through suppression of PI3K/AKT activation pathway) to GC T_{FH} cells, preventing excess CD4⁺ T cells proliferation that could derive from long-term TCR stimulation during prolonged T-B interaction in GCs. Absence of PD-1 is associated with reduced IL-21 and IL-4 secretion²³.

B and T lymphocytes attenuator (**BTLA**) is an inhibitory receptor expressed by B and T cells that binds herpes virus entry mediator (HVEM) and inhibits T cell proliferation. BTLA is upregulated on T_{FH} cells while is stable or downregulated on many other activated CD4⁺ T cells. BTLA deficiency is not associated to altered T_{FH} cell differentiation but with increased IL-21 production by T_{FH} cells²⁴. Tumor necrosis factor receptor superfamily member 4 (TNFRSF4), or **OX-40**, is a secondary co-stimulatory molecule, expressed after 24 to 72 hours following activation on T cells. OX40–OX40L interactions between CD4⁺ T cells and DC seem to be important for the up-regulation of CXCR5 and homing of CD4⁺ T cells to the follicle²⁵.

The importance of these molecules in T_{FH} function is underscored by the findings that targeted deletion of *Cd40/Cd40l*, *Icos*, *Il21/Il21r*, or *Sh2d1a* in mice, or loss-of-function mutations in *ICOS*, *CD40L*, and *SH2D1A* in humans severely compromise the generation of robust GC

reactions and subsequent long-lived serological memory against TD Ags²⁶ (Table 1).

T_{FH} cells express also a unique expression profiles of cytokines and soluble factors, such as high levels of IL-21, that drives STAT3-dependent plasma cell differentiation of B cells, IL-4, that has a strong anti-apoptotic activity on B cells and also C-X-C motif ligand 13 (CXCL13), a chemokine expressed only by human and not mouse T_{FH} cells, that attracts CXCR5⁺ B cells of secondary lymphoid organs to the T-B border zone (Table 2 and Fig. 3). T_{FH} and germinal center (GC) T_{FH} cells are two stages of a stepwise progression and have similar gene expression profiles, with GC T_{FH} cells being a further polarized state of T_{FH} cells, that express T_{FH}-specific molecules at a higher level (Table 2).

Table 2: Markers for human and mouse T_{FH} cells¹⁴

Marker	Human		Mouse		Naive CD4 T cell	Activated non-T _{FH} CD4
	T _{FH}	GC T _{FH}	T _{FH}	GC T _{FH}		
CXCR5	+	++	+	++	—	— ^a
Bcl6	+	++	+	++	—	—
PD-1	+	++	+	++	—	variable
ICOS	+	++	+	+	—	variable
SAP	normal	high	normal	high	normal	normal
IL-21	+	++	+	++	—	variable
CXCR4	normal	high	high mRNA	high mRNA	normal	normal
CXCL13	low	high	—	—	—	—
BTLA	normal	high	high	high	normal	low
SLAM	unknown	unknown	normal	normal	normal	variable ^b
CCR7	low	low	low	low	high	variable
CD200	—/+	+	+	+	—	—
IL-4	—	+	—	+	—	Th2 ⁺

^a*In vitro* activated human, but not mouse, CD4⁺ T cells transiently upregulate CXCR5 upon strong TCR + CD28 signaling.

^bSLAM expression is high on most T_{H1} and T_{H2} cells.

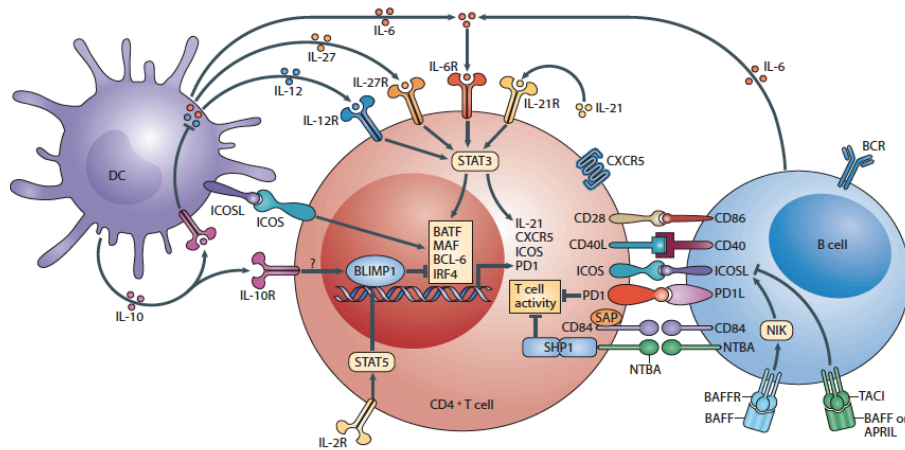


Figure 3: molecular mechanisms regulating T_{FH} cell function².

Surface receptors, cytokines, intracellular proteins and transcription factors involved in T_{FH} cell ability in providing help to B cells. During CD4⁺ T cell priming, DC secrete a specific cytokine cocktail (most of all IL-12, IL-21 and IL-27) that stimulate the expression, in a STAT3-dependent manner, of the transcription factors BCL6, MAF, basic leucine zipper transcriptional factor ATF-like (BATF) and interferon-regulatory factor 4 (IRF4), which then imprint the T_{FH} cell fate on a T cell by inducing the transcription of signature genes, including *CXCR5*, inducible T cell co-stimulator (*ICOS*), *IL21* and programmed cell death protein 1 (PD-1). CD28–CD86, CD40L–CD40 and ICOS–ICOSL interactions are central to this process. In addition, signalling lymphocytic activation molecule (SLAM) family receptors allow the recruitment of SLAM-associated protein (SAP) to these receptors facilitates and maintains conjugate formation between T and B cells. PDL1, PD-1 ligand 1.

1.2 The transcription factor network driving T_{FH} cell differentiation

BCL6: B-cell lymphoma 6 protein (BCL6) was recently identified as a master regulator of T_{FH} cells differentiation^{27, 28}. T_{FH} cells express high levels of BCL6, and non-T_{FH} cells (activated antigen-specific CD4⁺ T_H cells that are not T_{FH}, i.e., T_{H1}, T_{H2}, T_{H17}, or other subsets) express high levels of another transcription factor, the B-lymphocyte-induced maturation protein 1 (BLIMP1)^{27, 29, 30}. BLIMP1 and BCL6 are mutually antagonists^{29, 30} and CD4⁺ T cells that constitutively express BLIMP1 suppress the expression of BCL6 and fail to generate T_{FH} cells⁶. Contemporary, BCL6 is able to inhibit the differentiation of other CD4⁺ T cell subsets, by antagonizing transcription factors important for T_{H1}, T_{H2} or T_{H17} differentiation: specifically the transcriptional repressor BCL6 can directly bind the *TBX21* and human *RORC* promoter^{28, 31} and it's also able to downregulate GATA-3 expression³². The *BCL6* proto-oncogene encodes a POZ-zinc finger transcription factor that acts as a sequence-specific repressor of transcription through recruitment of a silencing mediator for retinoid and thyroid hormone receptors (SMRT) and histone deacetylase-containing complex³³. The *BCL6* gene is located at the telomeric extremity of chromosome 3 and spans 26 kb. The BCL6 protein is a 706 amino acid transcription repressor composed of an amino-terminal BTB domain (bric-a-brac, tramtrack, broad complex), also called Pox virus zinc (POZ finger) domain, a middle portion composed by three peptide sequences enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) domains (PEST), and

six zinc finger (ZF) domains at the carboxyterminus³⁴ (Fig. 4). These domains regulate transcription of target genes via distinct interactions, by recruiting class I and class II histone deacetylases complexes directly or through co-repressors. The homodimeric and heterodimeric BTB/POZ domain, displays a conserved protein–protein interaction motif, and is required for the repressive activity of the protein. This domain recruits several corepressors complexes, including NCOR1, NCOR2 (SMRT), CTBP1, MTA3 and BCOR, which in turn associate with both mSIN3A and histone deacetylases (HDACs) to constitute a large repressing complex³⁴. In addition, the BTB domain interacts with several other proteins which are members of the BTB/POZ-zinc finger family (BAZF, LRF, PLZF). The middle portion of BCL6, composed by three PEST domains, is involved in the protein activity and stability: it is an additional autonomous trans-repression domain, which interacts with MTA3 and CTBP1, and this domain is also essential for post-transcriptional regulation of BCL6 function through acetylation of the three PEST domains (through p300) (Fig. 5). The BCL6 ZFs bind DNA in a sequence specific manner, leading to the transcription repression of target genes. The highest affinity site has a core sequence of 9 bp (TTCCT(A/C)GAA) which has perfect homology with the consensus signal transduction and activators of transcription (STAT) binding site³⁴. This enables BCL6 repression of transcription via STAT factor binding sites and inhibits cytokine-induced transcription. In addition, the ZF region of BCL6 can specifically bind to Jun proteins (c-Jun, JunB and JunD, known as AP-1 factors) and regulate genes that do not have BCL6 DNA binding

sites in their promoters, such as BLIMP1 or p21cdk1. Other targets are depicted in Fig. 5.

Initially, BCL6 function had been mostly associated with B cell fate within the B lineage, where BCL6 expression is largely confined to GC B cells³⁵. In these cells, BCL6 represses genes that function in differentiation, inflammation, apoptosis and cell cycle control. BCL6 also autoregulates its transcriptional expression and up-regulates, indirectly, the expression of some genes important for GC reactions, such as activation-induced cytidine deaminase (AICDA), through the repression of microRNAs expression, like miR-155³⁶. *Bcl6*^{-/-} mice lack GCs and as a consequence display defective T cell-dependent antibody responses and demonstrate no antibody affinity maturation^{37, 38}. In 2009 it has been demonstrated that in the absence of BCL6 (*Bcl6*^{-/-}), T_{FH} differentiation does not occur *in vivo*^{27, 28} and BCL6 has been defined as master transcription factor of T_{FH} cells. Differentiation of other CD4⁺ T cell subsets is relatively unaffected by the loss of BCL6^{27, 28} but at the opposite BCL6 is both necessary and sufficient for T_{FH} cell differentiation: in the multistage and multifactorial process of T_{FH} cell differentiation, BCL6 is initially upregulated at the DC priming stage, its expression is stabilized at the T-B interaction phase and then there is a maximization of BCL6 expression in the GC phase¹⁴. Early after immunization BCL6 expression is detectable in a fraction of antigen-engaged T cells in the T-cell zone where majority of DCs exist³⁹. The most important signals for BCL6 upregulation in T_{FH} cells are TCR engagement together with costimulation through CD28 and ICOS, signals that are provided by cognate interactions of T_{FH} cells with DCs^{21, 40}. BCL6 upregulation is also promoted by IL-6

and IL-12-DC secreted cytokines. Important consequences of BCL6 overexpression are upregulation of CXCR5 and downregulation of CCR7^{28, 31}: the opposite modulation of these chemokine receptors is required for the correct localization of BCL6-expressing T cells into the T-B cell areas of lymphoid organs. After downregulating CCR7, T_{FH} cells downregulate also P-selectin ligand glycoprotein-1 (PSGL1), in a BCL6-dependent manner⁴¹: PSGL1 binds CCL19 and CCL21, cytokines normally present in T cell zones of secondary lymphoid organs, thus its downregulation facilitates the correct T_{FH} cells movement into follicles⁴². BCL6 expression in T_{FH} cells precedes that of B cells by 1-2 days and in B cells BCL6 mediates the downregulation of EBI2 (GPR183), that is important for the movement from the outer- and interfollicular-regions to the center of the follicle^{43, 44}. These data suggest that the main function of BCL6 in T_{FH} cell differentiation is to facilitate cognate interaction with B cells at the T cell zone. After DC priming, T_{FH} cells need B cells to sustain BCL6 expression and to promote the entry into GCs and T_{FH} cells maintenance. The stability of cognate T-B interaction is enhanced by interactions of the SLAM family receptors and the following activation of the small adaptor protein SAP (encoded by *SH2D1A*) that couples to the SLAM family receptors and promotes the intracellular signalling pathways. In the absence of B cells, BCL6 expression was not sustained and T_{FH} cells number decrease within the first week after immunization. In addition, in response to CD40 signals, B cells that downregulated ICOSL after activation, re-express it at higher levels on their surface and so ICOS-ICOSL interactions together with stromal-derived cytokines IL-6 and IL-21 contribute to

T_{FH} cells maintain and promote BCL6 expression. Thus, stable interactions between T and B cells after DC priming enable sustained expression of BCL6 in T_{FH} cells (Fig. 6).

T_{FH} cells can be distinguished from non-T_{FH} subsets by their pattern of cytokine production: human GC T_{FH} cells are the highest producers of IL-21 and the lowest producers of IFN- γ , IL-4 and IL-17, that is probably explained by the known ability of BCL6 to regulate the expression of T-bet (*TBX21*) transcription^{28, 31}, GATA-3 protein expression³² and ROR γ t expression^{28, 31} in humans and function in mice. Thus, BCL6 is important both to direct T_{FH} cells toward and inside the follicles and to modulate their function by limiting their ability to secrete proinflammatory cytokines inside GCs. The main and key function of T_{FH} cells, the provision of IL-21 and CD40L-mediated signals to B cells, is not exclusively dependent on BCL6 expression: IL-21 expression is mediated by *BATF*⁴⁵ and *c-MAF* signalling and until now a role in for BCL6 in the regulation of CD40L expression has not been demonstrated yet.

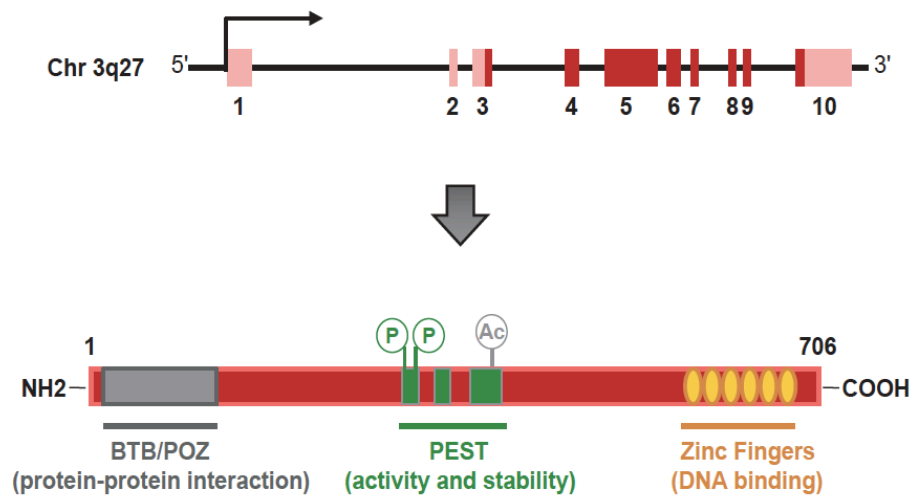


Figure 4: BCL6: gene, protein, and expression⁴⁶. (Top) Schematic representation of the *BCL6* gene. The non-coding and coding exon sequences are depicted in light and dark red, respectively. (Bottom) Schematic representation of the BCL6 protein. The three main functional domains (BTB / POZ, PEST, and ZF) are highlighted.

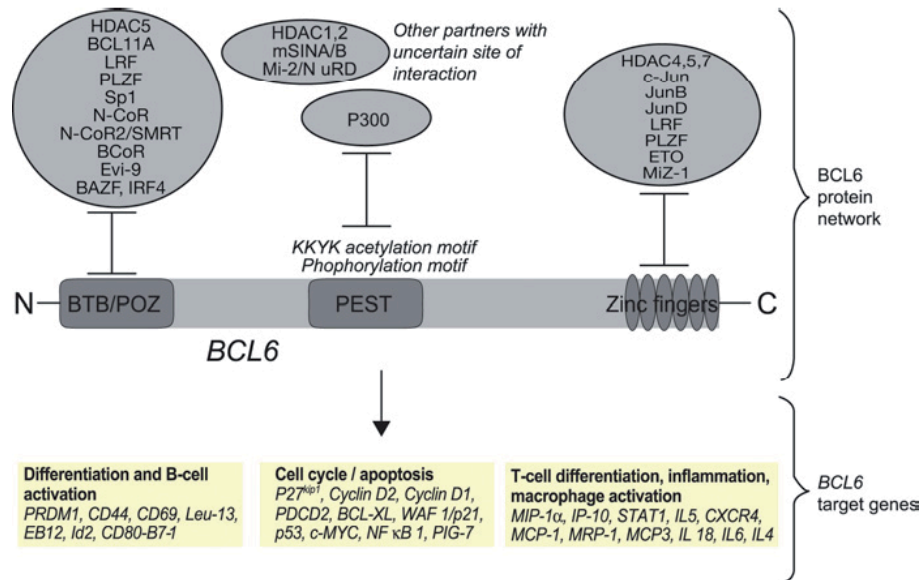


Figure 5: BCL6 interacting proteins and target genes³⁴: The diagram shows the conserved motifs that mediate BCL6 protein interactions. BCL6 directly interacts with several proteins via the POZ/BTB domain, the ZF domain, or both. p300 cofactor binds the PEST domain and acetylates BCL6, targeting it for repression. Engagement of BCRs by antigen induces MAP kinase mediated phosphorylation of BCL6, leading to BCL6 degradation by the ubiquitin proteasome pathway. Target genes are bound in a sequence specific manner by the BCL6 ZF. The ZF region of BCL6 can specifically and directly bind to jun proteins and represses genes that do not have BCL6 DNA binding sites in their promoters

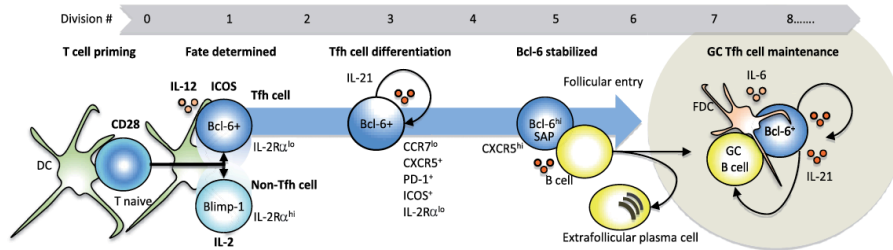


Figure 6: T cells primed by DCs can express BCL6 soon after CD28-mediated priming under the influence of cytokines such as IL-12 in humans⁵. Polarization into Bcl-6 versus Blimp-1-expressing cells can be observed as early as after the first cell division. Differentiation into T_{FH} cells (CCR7^{lo} CXCR5⁺ PD-1⁺ ICOS⁺ IL-2Rα^{lo}) occurs soon after that, prior to B cell interaction, and is enhanced by ICOS signaling. By contrast, IL-2 signaling promotes BLIMP1 expression in non-T_{FH} cells, which upregulate IL-2Rα expression. BCL6 expressing T_{FH} cells prime B cells for both GC and extrafollicular plasma cell responses. Cognate interaction with B cells stabilizes BCL6 expression, possibly coinciding with a reported “second wave” of BCL6 expression by T_{FH} cells at their fifth cell division. Highly and stably expression of BCL6 in T_{FH} cells is important for optimal downregulation of CCR7 and PSGL-1 and upregulation of CXCR5 and SAP, all of which are required for follicular entry and “residence” as GC T_{FH} cells. Within germinal centers, GC T_{FH} cells provide GC B cells that have taken up antigen held on follicular dendritic cells (FDCs), with growth and survival signals such as IL-21. T_{FH}-derived IL-21 and/or IL-6 of stromal origin also appear to be important for maintaining T_{FH} cells.

It appears that most cell types can be distinguished by differential expression of a set of 4–6 transcription factors (TFs). For T_{FH} cells, several transcription factors are required in addition to BCL6. These include MAF, interferon regulatory factor 4 (IRF4), the activator protein 1 (AP-1) family member “basic leucine zipper transcription factor” (BATF), signal transducers and activators of transcription STATs, and E proteins⁴⁷ (Fig. 3 and Fig. 7).

MAF: MAF (*c-MAF*) specifically regulates IL-4⁴⁸ and IL-21 expression⁴⁹, cytokines that are major products of GC T_{FH} cells. *c-Maf*^{-/-} chimeric mice have defective T_{FH} differentiation *in vivo*, due to lack of IL-21 production in an ICOS-dependent manner⁵⁰. MAF interacts with NFAT and JunB to induce IL-4 expression and directly binds to the IL-21 promoter. IL-4 has an anti-apoptotic activity on B cells (through induction of BCL-XL) while IL-21 is the most important known cytokine for T cell help to B cells, both through maintenance of GCs and for induction of plasma cell differentiation. In their study, *Kroenke et al.*¹³ found that BCL6 has minimal influence on IL-21 production, whereas MAF drives IL-21 production. Intriguingly, co-expression of BCL6 and MAF exhibited additive effects on gene expression for some of T_{FH} associated genes (PD-1 and ICOS), whereas in other cases BCL6 and MAF appear to have parallel, nonadditive capacities (e.g., CXCR5). Altogether, these findings reveal that BCL6 and MAF collaborate to orchestrate a suite of genes that define nearly all of the core characteristics of human T_{FH} cell biology¹³.

IRF4 and BATF are both essential for T_{FH} cell differentiation, but they are required for multiple different CD4⁺ T cell programs and can be

considered early T cell activation-associated transcription factors that enable expression and function of downstream cell-fate-determining transcription factors^{49, 51}.

BATF: BATF (basic leucine zipper transcription factor, ATF-like) is an AP-1 superfamily member capable of partnering with Jun to control lymphoid development and differentiation. Batf-deficient mice were recently reported to have defects in T_{H17}⁵², T_{H2} and T_{FH}⁵¹ development. BATF also regulates GC development, including both T_{FH} and GC B-cell generation^{45, 51, 52}. In T_{FH} cells, BATF was shown to control BCL6 and c-MAF expression, while in B cells, BATF regulates class switching recombination (CSR) events through modulating the expression of activation-induced cytidine deaminase (AID) and germline transcription (GLT)⁴⁵.

IRF4: Mammalian interferon regulatory factor (IRF) family members play multiple roles within and beyond the immune system^{53, 54}. Many of IRF members are expressed in T cells and play important roles in regulating T-cell differentiation⁵³. IRF4, a weak DNA binder, is expressed specifically and uniquely in cells of the immune system. It needs cofactors for stable binding with chromatin, in order to perform its transcriptional regulation function: IRF4 can interact with BATF-JUN complex in controlling transcriptional regulation^{55, 56}. In B cells IRF4 is frequently found in cooperation with STAT3, regulating IL-21-induced genes, including BLIMP1 (*PRDMI*)⁵⁷. *Irf4*^{-/-} CD4⁺ T cells in mouse exhibit diminished STAT3 binding and as consequence possess defective T_{FH} cells⁵⁷, due to a T-cell-intrinsic defect⁵⁸. In general IRF4 can interact with BATF-JUN complex in several cells of the immune system and an IRF4 deficiency causes an impairment in

differentiation of several CD4⁺ T cells (T_{H1}, T_{H2}, T_{H9}, T_{H22}), together with T_{FH} cells.

E proteins: The E protein Ascl2 provides a newly recognized contributor to T_{FH} differentiation⁵⁹. Constitutive expression of Ascl2 in murine CD4⁺ T cells induces CXCR5 expression by some CD4⁺ T cells *in vitro*⁵⁹. Similar to BCL6, Ascl2 acts as a novel suppressor of T_{H1}, T_{H2} and T_{H17} differentiation and as unique regulator of T_{FH} cells development and function by increasing CXCR5 and CXCR4 expression and suppressing CCR7, PSGL1 expression and IL-2 signalling. These data indicates that in mouse Ascl2 and Bcl6 act to program T_{FH} cell generation, but further investigations should unveil ASCL2 role in human T_{FH} cells.

STATs: Signal transducers and activators of transcription (STATs) are also deeply entwined with T_{FH} differentiation, as they are involved with all CD4⁺ T cell differentiation pathways⁶⁰. Roles of STAT proteins in T_{FH} cells are complex and overlapping. STAT3 is the most important STAT in murine T_{FH} differentiation⁶¹, while STAT1 and STAT4 can also contribute. STAT3 has a critical role in IL-21 expression by murine CD4⁺ T cells⁶², whereas both STAT4 and STAT3 regulate IL-21 expression by human CD4⁺ T cells^{61, 63, 64}. Tangye's group⁶¹ reported that STAT3 deficiency compromised the generation of human T_{FH} cells as well. Mutation in STAT3 abolished IL-6/IL-21/IL-12-induced expression of IL-21 by human T cells, which resulted in diminished T_{FH} cell generation and abolished B-cell helper activity *in vivo*. Thus, STAT3 functions as a critical factor for mouse and human T_{FH} cell differentiation⁶¹. In contrast a constitutive active form of STAT5 (activated by IL-2 signalling) efficiently

inhibits T_{FH} differentiation by suppressing the expression of T_{FH}-associated genes such as *CXCR5*, *c-MAF*, *BCL6*, *BATF*, and *IL21*, whereas STAT5 deficiency greatly enhances T_{FH} gene expression *in vitro*^{65, 66} (Fig. 7).

In summary, for the T_{FH} cell program, BCL6, MAF, STAT3 and STAT4 (only STAT3 in mouse) are the core transcription factors that control four general aspects of T_{FH} cell biology: location, function, differentiation or positive reinforcement. The fourth important attribute is repression of alternative cell-fate differentiation programs. BCL6 directly represses cytokine receptors and transcription factors necessary for T_{H1}, T_{H2}, T_{H17} cell differentiation, and also represses BLIMP1, which is strongly associated with non-T_{FH} cell fates^{27, 67}.

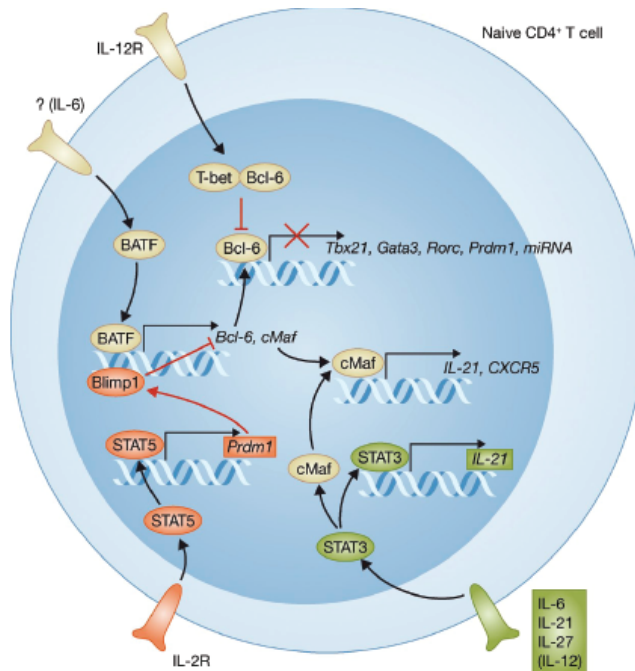


Figure 7: Molecular regulation of T_{FH} development⁶¹. T_{FH} development requires the integrated and balanced function of numerous transcription factors. BATF acts proximally by inducing BCL6 and MAF. BCL6 imprints a T_{FH} fate by suppressing expression and/or function of transcriptional regulators of alternate effector fates (i.e., *TBX21* (T_{H1}), *GATA3* (T_{H2}), and *RORC* (T_{H17})), microRNAs that suppress T_{FH} generation, and *PRDM1* (encoding BLIMP1), which directly suppresses BCL6. c-MAF contributes to T_{FH} cells by inducing CXCR5 and IL-21. STAT3-activating cytokines IL-6, IL-21, and IL-27 can also induce c-MAF, and STAT3 can directly induce IL-21. IL-12 induces IL-21 in a STAT3-dependent manner, but it is unknown whether this involves c-Maf or is a direct effect of STAT3. T_{FH} generation is restricted by BLIMP1, which is induced by IL-2 in a STAT5-dependent manner and blocks BCL6 expression, and by T-bet (induced by IL-12), which physically associates with BCL6 and prevents BCL6 dependent repression.

1.3 T_{FH} cells differentiation

T_{FH} cells differentiation is a multistage, multifactorial process, because there is no single event that defines the differentiation of these cells. This multistage differentiation model, proposed by *Crotty S.*^{14, 67} and confirmed by several studies, is composed by three main stages: dendritic cells (DC) priming, commitment (T-B border phase) and full polarization (GCs phase) (Fig. 8).

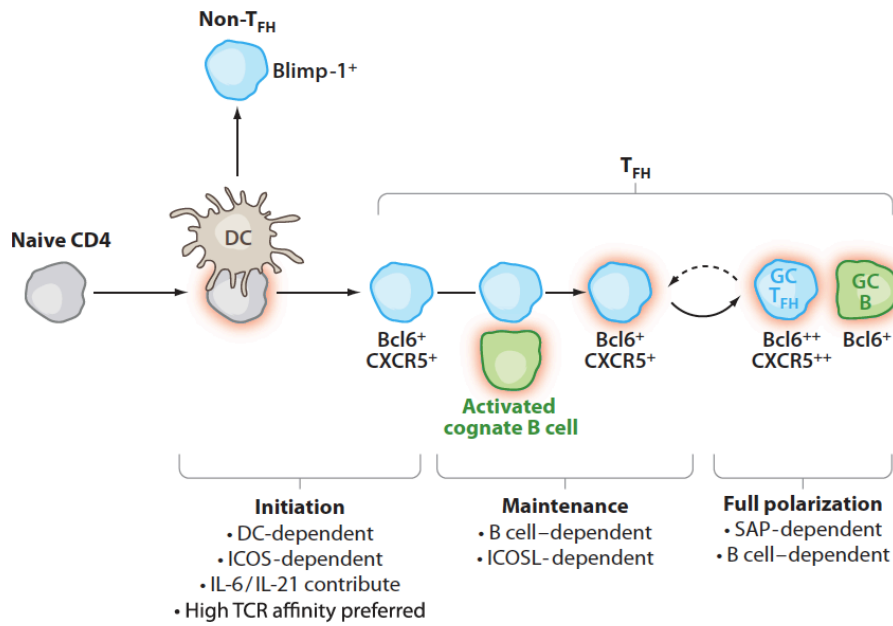


Figure 8: Proposed integrated model of T_{FH} differentiation: multistage and multifactorial¹⁴. Multiple stages of T_{FH} differentiation, including initiation, maintenance, and full polarization.

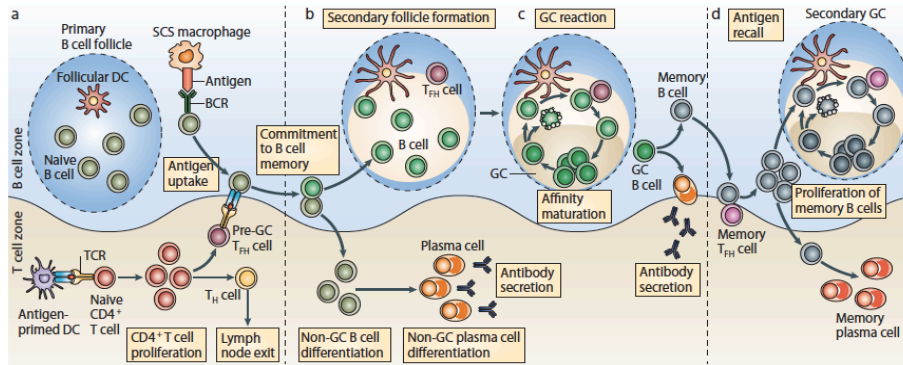


Figure 9: T_{FH} cell differentiation⁶⁸: **(a)** Local protein vaccination or pathogen entry induces dendritic cell (DC) maturation and migration to the T cell zones of draining lymph nodes. DCs that express peptide–MHC class II complexes engage naive, antigen-specific CD4⁺ T cells to induce their proliferation and differentiation into effector T helper (T_H) cells. In the B cell zone, antigen-specific B cells become activated, take up, process and present antigenic peptides and migrate towards the B cell–T cell borders of the draining lymph node. Emigrant T_H cells exit the lymph node to function at distal tissue sites and T follicular helper (T_{FH}) cells relocate to B cell–T cell borders and interfollicular regions. Cognate contact between pre-germinal centre (pre-GC) T_{FH} cells and antigen-primed B cells is required for multiple programming events in the pathway to B cell memory. **(b)** Clonal expansion, antibody class switching and non-GC plasma cell development proceeds in the extrafollicular regions of the lymph nodes. **(c)** Polarization of the secondary follicle anatomically signifies the initiation of the GC cycle. The dark zone supports GC centroblast proliferation, class-switch recombination and B cell receptor (BCR) diversification through somatic hypermutation. Non-cycling GC centrocytes move to the light zone and continually scan follicular DC networks. Centrocytes that lose the ability to bind to the presented antigen undergo apoptosis, while those that express a variant BCR with a higher affinity can compete for binding to antigen-specific GC T_{FH} cells **(d)** Following antigen re-challenge, memory B cells present antigens to memory T_{FH} cells to promote memory B cell clonal expansion with rapid memory plasma cell generation and the induction of a secondary GC reaction.

The canonical T_{FH} cell differentiation process starts with the initial dendritic cell (DC) priming of a naive CD4⁺ T cell that from blood has migrated to the T cell zone of secondary lymphoid organs. The CD4⁺ T cell undergoes a cell-fate decision within the first few rounds of cell division. If the chemokine receptor CXCR5 is expressed, the early T_{FH} cell will migrate to the border of the B cell follicle and undergo further T_{FH} cell differentiation. If the cell instead receives T_{H1}, T_{H2}, or T_{H17} signals, then the CD4⁺ T cell follows a T_{H1}, T_{H2}, or T_{H17} cell differentiation program, including upregulation of chemokine receptors for inflammatory chemokines that will drive the effector cell to exit the lymphoid tissue and traffic to the site of infection or inflammation (Fig. 9a).

Early T_{FH} cell differentiation (**the DC priming phase**) is regulated by IL-6, ICOS, IL-2, and TCR signal strength, as shown in mouse models (Fig. 8 and 9a). Higher TCR affinity has been associated with a preference for T_{FH} differentiation over non-T_{FH} differentiation⁶⁹, which may be due to higher TCR affinity causing extended T-DC interactions and resulting in prolonged costimulatory receptor engagement and/or cytokine exposure at the time of DC priming. ICOS expression by CD4 T cells at the time of DC priming is required for expression of BCL6 and T_{FH} differentiation. ICOS is upregulated on all helper T cells after CD28 priming. Expression of ICOSL on DCs appears to be necessary to induce the T_{FH} cell programme over more typical effector T helper cell options²¹: ICOS-ICOSL interactions activate the phosphoinositide 3-kinase (PI3K) pathway that support a strong secretion of T_{FH} effector cytokines IL-21 and IL-4, and also induces MAF expression and the upregulation of BCL6²¹.

DCs are able to produce IL-6 in mice and also IL-12 in humans¹⁰ that promote T_{FH} cell formation *in vitro*. IL-6 is the earliest non-TCR signal involved in initiation of T_{FH} cell differentiation and IL-6 signaling transiently induces BCL6 expression by newly activated CD4⁺ T cells³¹. The upregulation of BCL6 causes the upregulation of CXCR5, that binds chemokine CXCL13 secreted by both human T_{FH} themselves and by follicular dendritic cells (FDCs), that together with the downregulation of CCR7 allow the localization of T_{FH} cells at the interface of the T zone and the B-cell follicle (T-B border) of secondary lymphoid organs (Fig. 9a). T_{FH} cells also downregulate P selectin glycoprotein ligand 1 (PSGL1), which anchors T cells to CCL19 and CCL21, present in the T cell zone extracellular matrix⁴¹. While CD4⁺ naive T cells are performing DC priming in order to start their T_{FH} cell differentiation after infection or immunization, naive B cells that have entered the secondary lymphoid organs (i.e. spleen or lymph nodes) can bind soluble antigen or can encounter their specific antigen on the surface of FDCs in the B cell zone (follicle) (Fig. 9a). If the B cell does not encounter antigen, it will remain in the secondary lymphoid tissue for a day and then migrate through the circulation to another secondary lymphoid tissue. Those B cells that bind antigen will become activated, internalize the antigen, and upregulate the chemokine receptor CCR7, whose ligands CCL19 and CCL21 are expressed in the adjacent T zone, and migrate to the (T-B border)^{70, 71}. Activated B cells also upregulate another migratory receptor, EBI2 (GPR183), that responds to 7 α ,25-dihydroxycholesterol and facilitates the distribution of B cells along the T-B border⁷². Here, the B cells can present fragments of peptide

on major histocompatibility complex (MHC) class II to cognate T cells that have been previously primed by DCs with the same antigen encountered by B cells and that have migrated to the T-B border by decreasing the expression of CCR7^{6, 73} (Fig. 9a). During this interaction B cells receive help from T_{FH} cells and become activated. These activated B cells localize to the perimeter of the follicle and the cognate interaction between T and B cells is crucial both for T_{FH} cells and for B cells differentiation, and is called the commitment phase or pre-GC phase (Fig. 9b and Fig. 10). Although CXCR5 expression is sufficient for localization to the T-B border, CXCR5 expression is not sufficient for T_{FH} localization inside follicles⁷⁴. The migration of T_{FH} cells to the T-B border allows the critical B cell-dependent phase of T_{FH} differentiation to occur. Activated B cells express ICOSL, and the expression of ICOSL by B cells is required for T_{FH} cells maintenance⁷⁵. BCL6 expression in T cells induced by interactions with DCs is short-lived, while cognate interactions between T and B cells are essential for the maintenance of the BCL6⁺CXCR5⁺ and B cells become both APC and a source of ICOSL for T_{FH} cells. Activated B cells then transiently localize to the perimeter of the follicle where they divide before taking on one of two fates: differentiation into short-lived extrafollicular plasma cells or initiation of a GC response^{6, 73} (Fig. 9b and 9c). The precursors to extrafollicular plasma cells migrate from the perimeter of the follicle to the medullary cords in the lymph nodes or the splenic junction zones (also known as bridging channels) to form extrafollicular foci. At these sites, plasmablasts associate with CD11c^{high} DCs that support their survival and differentiation into plasma cells. Extrafollicular plasma

cells will secrete class-switched antibodies for 3 days, after which the majority undergo apoptosis⁷⁶. A small fraction can live for longer periods in the secondary lymphoid tissues. This initial burst of immunoglobulin with modest affinity for antigen produced in the extrafollicular zones plays an important role in the early control of infection. An alternative fate for B cells that have localized to the perimeter of the follicles after the first cognate interaction with T cells is to migrate toward the center of the follicle (mediated by downregulation of EBI2) and seed the GC response (Fig. 9c). Human pre-GC T_{FH} and GC T_{FH} cells, together with FDCs and macrophages, are also able to secrete increasing amounts of CXCL13, that is CXCR5 ligand and allows the movement of B cells to the T-B border of the secondary lymphoid organs.

Reporter mouse models have revealed that T cells produce cytokines at sites of antigen-specific contact with cognate B cells, and T cell-derived cytokines can be visualized in the follicular regions following initial T cell contact with B cells: IL-4 promotes IgG1 and IgE class switching⁷⁷; interferon- γ (IFN γ) induces IgG2a⁷⁷; and transforming growth factor- β (TGF β) directs commitment to IgA⁷⁸. In this manner, pre-GC B cell-T_{FH} cell contact, involving the delivery of different cytokines, can imprint antibody class switching among the progeny of the antigen-responsive B cells.

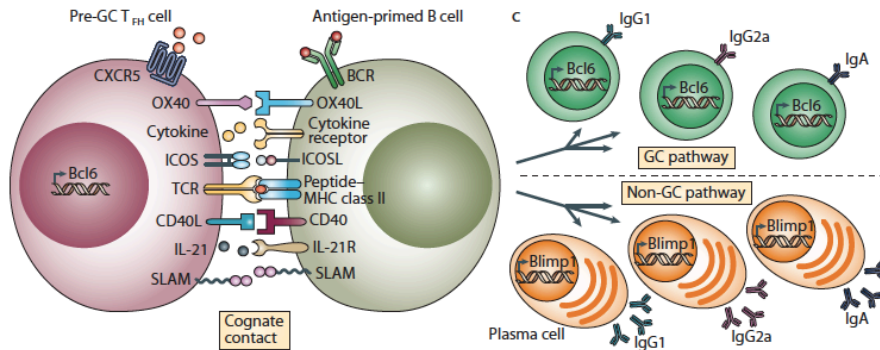


Figure 10: pre-GC phase: commitment to memory⁶⁸. Antigen-primed B cells must process and present peptide–MHC class II complexes to receive cognate help from pre-GC T_{FH} cells. Upregulation of the molecules involved in T_{FH} cell contact is a poorly resolved component of early antigen-driven B cell maturation. Cognate contact between antigen-specific TCRs and peptide–MHC class II complexes focuses the intercellular exchange of molecular information between pre-GC B cells and T_{FH} cells. The modifying interactions that occur at first contact are known to involve co-stimulatory molecule interactions (for example, CD40L–CD40 and ICOS–ICOSL), accessory molecule interactions (for example, SLAM family interactions and OX40–OX40L) and interactions between cytokines and their receptors (for example, interleukin-4 (IL-4)–IL-4R, interferon- γ (IFN γ)–IFN γ R and IL-21–IL-21R). The distribution of these functional attributes in pre-GC T_{FH} cell compartments is not yet well resolved *in vivo*.

1.3.1 The GC reaction

Germinal centers (GCs) are dynamic microanatomical structures that arise in the follicular regions of secondary lymphoid tissues to support the generation of high-affinity B cell memory: B cell entry into the GC reaction is regulated by antigen-specific T_{FH} cells (Fig. 9c and Fig. 11). Two phenotypically distinct populations of BCL6-expressing T_{FH} cells are readily identifiable in mice and humans. These are referred to as pre-T_{FH} and T_{FH} cells, or T_{FH} and GC T_{FH} cells in different nomenclatures. GC T_{FH} cells are further differentiated T_{FH} cells that express the highest levels of BCL6 and CXCR5 in mouse and human tonsillar CD4⁺ T cells (Bcl6⁺⁺CXCR5^{hi})^{79, 80}. In humans GC T_{FH} cells are most frequently identified through the highest CXCR5 and ICOS expression¹⁴. These markers identify GC T_{FH} cells as T_{FH} cells within GCs, whereas the remaining T_{FH} cells are predominantly in the follicle or in apposition to the follicle^{79, 80}. Adhesion molecules play an important role on GC T_{FH} cells, regulating their interaction with GC B cells and their localization. The stability of cognate T-B cells interactions is the key for the T_{FH} cells pool maintenance and for the GCs formation¹⁵. This interaction is mediated by signaling lymphocyte activation molecule receptors (SLAM) and by SLAM-associated protein (SAP). SAP is indispensable for the GC development because it is critically required for stable and prolonged formation of T-B conjugates, it is essential for IL-4 secretion and also modulates the crosstalk between SLAM family receptors and the TCR¹⁴. Prolonged T-B cell contact allows the continuous help of T_{FH} cells to B cells, through survival and differentiation signals (IL-21 and CD40-

CD40L) that promote the survival and differentiation of both T_{FH} cells (thank to ICOSL on B cells) and GC B cells and that starts the germinal center reaction. During the GC response B blasts move to the pole of the follicle closer to the T zones to form the dark zone, (after expressing high levels of CXCR4): these rapidly dividing cells are then known as centroblasts. Centroblasts express high amounts of the enzyme activation-induced deaminase (AID), which is essential both for antibody class-switch recombination and for the process of somatic hypermutation (SHM)³⁹. This process introduces mutations in the V-region genes of the B-cell receptor (BCR): the random nature of SHM results in some B-cell clones yielding higher-affinity BCRs whereas others lose reactivity all together or even become self-reactive. Selection of mutated GC B cells must therefore occur to ensure that those B cells with the highest affinity BCRs receive survival signals, whereas those expressing self-reactive receptors are eliminated⁷³: this selection is called affinity maturation. For selection to occur, centroblasts first exit cell cycle, differentiate into non-dividing centrocytes that express the mutated BCRs on their surface, and move toward the light zone⁸¹. The light zone is densely packed with centrocytes in close contact with FDCs. Centrocytes move over the FDC network, collecting antigen from the surface of FDCs. This antigen is internalized, processed, and presented on MHC class II to GC T_{FH} cells that also reside in the light zone^{6,7}. Without T-cell help in the GC, the response collapses and does not produce long-lived plasma cells or memory B cell. During the GC response, interactions with T cells are essential for the survival of GC B cells, because if this interaction doesn't occur (when the affinity of BCR is low or for self-

antigens), GC B cells die by apoptosis. B cells whose somatic mutations have improved the affinity of the BCR are able to capture more antigen and outcompete lower-affinity centrocytes for help from T_{FH} cells. Centrocytes that receive positive selection signals from T_{FH} cells can either exit the GC as long-lived plasma cells, memory B cells, or return to the dark zone where they can undergo further SHM⁸¹ (Fig. 9c and Fig. 11). Competition for limiting numbers of T_{FH} cells is critical to the process of affinity maturation and controls the output of the GC response⁸¹. Cell death is a prevalent outcome of the GC cycle. GC T_{FH} cells provide proliferation, survival and differentiation signals to centrocytes through CD40L and cytokines. CD40 is important for B cell activation, proliferation, and survival *in vitro* and *in vivo*⁸² because GC B cells are highly proapoptotic and require constant survival signals. T_{FH} cells express high levels of IL-21 and varying amounts of IL-17, IL-4 and IFN γ . IL-21 secreted by CD4⁺ T cells delivers important signals to B cells stimulating them to undergo proliferation and differentiation⁸³. T_{FH} cells can also deliver negative selection signals through engagement of CD95 (also known as FAS), which is highly expressed by human and mouse GC B cells. CD95 signalling limits the numbers of memory B cells, the accumulation of mutations in the memory B cell pool and long-term proliferation of GC B cell. At the same time GC T_{FH} cells express high levels of the inhibitory receptors BTLA and PD-1, that are important for controlling the size of GC T_{FH} cells pool¹⁵.

GC B cells are strictly confined to a single GC, and the majority of GC B cells within a GC represent oligoclonal antigen-specific B cell clones undergoing hypermutation and selection by T_{FH} cells. In

contrast, the GC T_{FH} cells can readily exit a GC and (1) transit to a neighboring follicle and enter a different GC⁸⁴, (2) temporarily reside in the adjacent B cell follicle before re-entering the same GC (Fig. 9d), or (3) exit a GC and downregulate BCL6 and develop into a memory T_{FH} cell.

Several groups reported a continuous scanning activity of GC B cells over follicular DC networks that were laden with immune complexes. After scanning and interact with follicular DCs, only a few GC B cells were shown to make stable, immune synapse-like contacts with GC T_{FH} cells (Fig. 11). GC B cells that were capable of presenting higher levels of antigen to T_{FH} cells exited the GC reaction rapidly and produced more post-GC plasma cells than GC B cells, that were less efficient at antigen presentation. Many of the molecules associated with pre-GC T_{FH} cell function may also function within the GC: BCL6 expression itself is reinforced in T_{FH} cells following contact with pre-GC B cell. ICOS-ICOSL interactions are important throughout this pathway, at the early DC contact and pre-GC contact stages and probably during the GC reaction itself. IL-21 and its receptor appear to be of continued importance at the pre-GC stage and during the GC reaction. Elevated expression levels of programmed cell death protein 1 (PD-1) correlate with GC localization of the T_{FH} cell compartment, and the absence of PD-1 ligand 2 (PD1L2) on B cells affects plasma cell generation and affinity maturation⁶⁸.

Two main B cell types emerge from GC reaction: memory B cells and plasma cells secreting high affinity antibody (Fig 9d). The population of non-immunoglobulin-secreting cells that is produced in the GC reaction during a primary response largely comprises class-specific

affinity-matured memory B cells. Affinity-matured IgM⁺ memory B cells can emerge from the GC reaction and persist for long periods *in vivo*⁸⁵. These non-switched memory cells appear to be more active in secondary responses in the absence of circulating antibodies. Antigen recall responses by memory B cells promote accelerated clonal expansion and rapid differentiation to high-affinity plasma cells⁶⁸. High-affinity antibody-producing plasma cells that emerge from the GC reaction can be considered an integral part of antigen-specific B cell memory. High-affinity GC B cells preferentially assort into the plasma cell compartment and produce high-affinity circulating antibodies. In the lymph nodes, affinity-matured plasma cells dwell in paracortical areas to mature and then migrate towards the medullary regions before export. After exiting from SLO, these plasma cells migrate through the bloodstream to find survival niche (predominantly located in the bone marrow), where they receive survival signals and they can continuously secrete high-affinity antibodies⁸⁶.

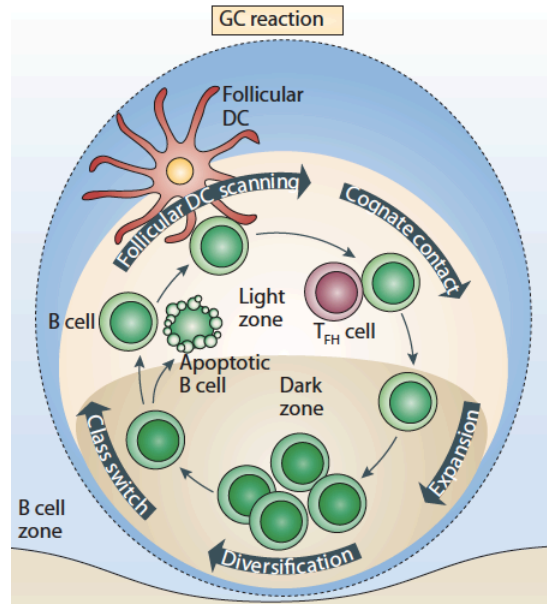


Figure 11: The antigen-specific GC reaction⁶⁸: The GC cycle is initiated through the pre-GC contact of B cells with cognate T_{HF} cells, as this promotes the extensive proliferation of antigen-primed B cells. The GC cycle is thought to begin when a secondary follicle polarizes to form two microanatomically distinct regions: the T cell zone-proximal dark zone (which contains proliferating centroblasts) and the T cell zone-distal light zone (which contains centrocytes, antigen-laden follicular DC networks and antigen-specific GC T_{HF} cells).

1.3.2 Cytokines important for T_{FH} cells differentiation

As with other helper T cell subsets, cytokine signals are important for the early development of T_{FH} cells. For these cells cytokines are provided mainly by dendritic cells during the priming phase. Recent studies show that the dominant cytokines associated with the development of T_{FH} cells differ between mice and humans. In mice, IL-6, IL-21 and IL-27 (which activate STAT3) have dominant roles^{14, 87}, with IL-6 that is a potent inducer of IL-21 and Bcl6, while IL-12 (which mainly activates STAT4) can also participate in the early phase but it's a poor inducer of IL-21⁸⁸. In humans, IL-12 induces higher expression of IL-21, ICOS, CXCR5 and BCL6 on activated naive helper T cells than IL-6, IL-21, and IL-27^{10, 64}. At the same time IL-6 is not a good inducer of IL-21, BCL6, or CXCR5 expression by activated human CD4⁺ T cells^{10, 64}. A recent paper reports the most successful conditions yet for human T_{FH} cell differentiation *in vitro*, with successful short term induction of CXCR5, BCL6, and IL-21 in the presence of transforming growth factor β (TGF- β) and IL-12 or IL-23⁶³. TGF- β contributes to the CD4⁺ naive T cells differentiation towards a T_{FH}-like phenotype by strongly enhancing STAT3 and STAT4 functions in inducing important T_{FH} molecules, including CXCR5, ICOS, IL-21, BCL6, BATF and MAF, and also suppressing the expression of BLIMP1, a transcription repressor that inhibits the function of BCL6⁶³. This is in stark contrast to mouse CD4⁺ T cells, in which TGF- β signals suppress expression of T_{FH} molecules including IL-21, ICOS and BCL6⁶³.

1.4 Memory T_{FH} cells in peripheral blood

A large population of CXCR5⁺ CD4⁺ T cells is present within the memory CD4⁺ T cell compartment (CD45RO⁺) in human peripheral blood, and that was described for the first time in 1994⁸⁹. Antigen-specific CD4⁺CXCR5⁺ T cells can be maintained in blood for many decades without restimulation, as showed in different studies on smallpox vaccinated subjects⁹⁰. CD4⁺CXCR5⁺ T cells contain long-lived memory cells and share functional properties with T_{FH} cells, so they are called blood (or peripheral) memory T_{FH} cells.

The generation and maintenance of effector T_{FH} cells require sustained antigen stimulation that keep high the BCL6 expression, so when the protein antigen is gradually cleared or no more available for T_{FH} cells, BCL6 protein is downregulated in these cells. CD4⁺CXCR5⁺ T cells in the blood do not express BCL6 protein, they lack expression of activation markers such as CD69 and ICOS^{4, 10} and have lower expression of the repression marker PD-1 compared to T_{FH} cells. Despite this, *in vitro*-cultured blood-derived CD4⁺CXCR5⁺ T cells produce more IL-21, IL-10 and CXCL13, which are all features of T_{FH} cells, and are more efficient at inducing B cell differentiation than CD4⁺CXCR5⁻ T cells^{4, 10}. A vast majority of memory T_{FH} cells express CD62L and CCR7, central memory markers, that allow them to go into and patrol secondary lymphoid organs⁹¹.

Blood memory T_{FH} cells constitute approximately 15-20% of memory CD4⁺ T cells in humans and are composed of heterogeneous cell populations with different phenotypes (Fig. 12). Blood memory T_{FH} cells can be subdivided by different approaches. The first set of parameters defines three major subsets: CXCR3⁺CCR6⁻ cells that

share properties with T_{H1} cells, expressing T-bet and secreting IFN- γ (called T_{FH1}), CXCR3⁻CCR6⁻ cells resembling T_{H2} cells, expressing GATA-3 and secreting IL-4, IL-5 and IL-13 (called T_{FH2}) and CXCR3⁻CCR6⁺ cells resembling T_{H17} cells, expressing ROR γ T and secreting IL-17A and IL-22 (called T_{FH17})⁹². The T_{H2} -and T_{H17} -like T_{FH} cell subsets were shown to produce higher levels of IL-21 and to induce naive B cell differentiation more efficiently than the T_{H1} -like T_{FH} cell subset. In contrast, blood memory T_{FH1} cells lack the capacity to help naive B cells^{92, 93}. Furthermore, whereas blood memory T_{FH2} cells promote IgG and IgE secretion, blood memory T_{FH17} cells induce IgG and IgA secretion⁹³. Thus, T_{FH2} and T_{FH17} cells represent efficient B cell helper cells with a distinct capacity to regulate immunoglobulin isotype switching. Another set of parameters define three other subsets: one activated subset (ICOS⁺PD-1²⁺CCR7^{lo}) and two quiescent subsets (ICOS⁻PD-1⁺CCR7^{int} and ICOS⁻PD-1⁻CCR7^{hi}). In healthy subjects ICOS expression is limited to a small population of blood T_{FH} cells; this population substantially increases after vaccination, and thus defines activated memory T_{FH} cells. ICOS⁻ cells are further divided into PD-1⁺ cells (~30% of blood memory T_{FH} cells) and PD-1⁻ cells, both of which lack the expression of Ki67 and thus are in a quiescent state^{92, 94}. CCR7 expression by blood memory T_{FH} cells negatively correlates with that of PD-1⁹¹. The matrix combination of the three parameters defines nine blood memory T_{FH} subsets. The activated ICOS⁺PD-1²⁺CCR7^{lo} populations within blood memory T_{FH2} and T_{FH17} cells might represent the most efficient helpers. Among quiescent T_{FH2} and T_{FH17} subsets, the ICOS⁻PD-1⁺CCR7^{int} population provides help to memory B cells more promptly

than the ICOS⁻PD-1⁻CCR7^{hi} population, but the ICOS⁻PD-1⁺CCR7^{int} and ICOS⁻PD-1⁻CCR7^{hi} T_{FH2} and T_{FH17} subsets are equally capable of helping naive B cells⁹² (Fig. 12).

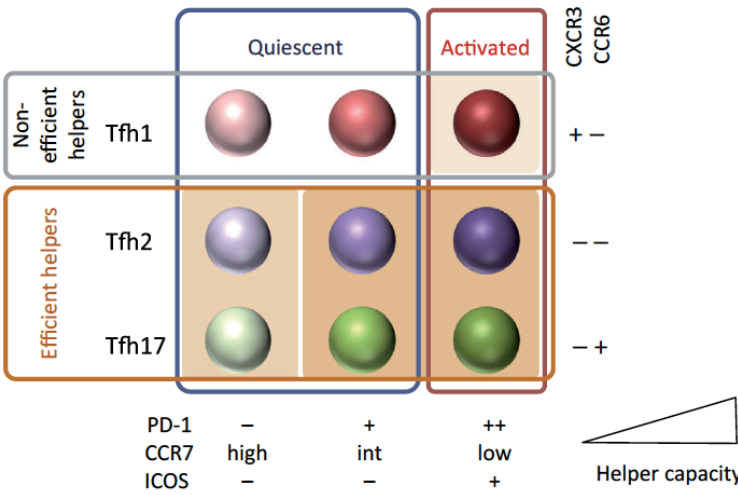


Figure 12: The nine blood memory T_{FH} cell subsets defined by a 3D analysis⁹¹. The nine blood memory T_{FH} subsets. The markers CXCR3 and CCR6 largely separate non-efficient helpers (T_{FH1}) and efficient helpers (T_{FH2} and T_{FH17}). ICOS expression defines quiescent subpopulations and activated cells in each subset. The helper capacity of T_{FH1} cells is limited to the activated ICOS⁺PD-1²⁺CCR7^{lo} subset that can help only memory B cells. Although both quiescent subsets within T_{FH2} and T_{FH17} cells are capable of helping B cells, the ICOS⁻PD-1⁺CCR7^{int} subset provides a prompt help to memory B cells. T_{FH2} and T_{FH17} cells produce different sets of cytokines and differentially regulate isotype switching. The intensity of the background orange color of each subset reflects the capacity to provide help to B cells.

1.5 Pathophysiology of follicular helper T cells

Since T_{FH} cells have a crucial and essential role in determining B cell differentiation into high-affinity plasma cells and memory B cells, regulating humoral immunity, mechanisms that control their numbers and activity are strongly needed in order to avoid dysregulation of humoral immune responses. T_{FH} cells control their high proliferation mainly by high expression of inhibitory receptors, such as BTLA or PD-1, that can control proliferation of these cells. Some recent works showed that a subset of thymic-derived T_{reg} cells is able to entry into follicles and to acquire typical T_{FH} cells phenotypical markers: they become $CXCR5^{hi}PD-1^{hi}ICOS^{hi}BCL6^{+}$, and they express at the same time typical T_{reg} markers, such as FOXP3, CTLA-4 and GITR and also high levels of IL-10. These FOXP3⁺ T follicular regulatory (T_{FR}) cells have the essential role of repressing T_{FH} cells activity and/or GC numbers, preventing the outgrowth of non-antigen specific B cells⁹⁵. Despite these mechanisms of control, several T_{FH} -related autoimmune diseases exist, due to an altered tolerance in GCs, that causes aberrant accumulation of T_{FH} cells and consequent autoantibody production and systemic autoimmunity. The molecular mechanisms causing dysregulated behaviour of $CD4^{+} T_{FH}$ cells and extrafollicular $CD4^{+} T_H$ cells, found to contribute to autoimmune in several mouse models of human disease, are still poorly understood. Moreover, several genetic defects in T_{FH} cells-key genes (i.e. ICOS, CD40L, SAP) severely impairs T_{FH} cells functionality and numbers and determine severe immune pathologies and primary immunodeficiencies⁹⁶.

1.5.1 T_{FH} cells in primary immunodeficiencies

Primary immunodeficiencies are caused by genetic defects that alter expression of molecules involved in immune system development and/or function. T_{FH} cells and their specific molecules are also involved in this process. Patients with ICOS deficiency suffer from common variable immunodeficiency (CVID): they show severely impaired GC formation in lymphoid tissues and severely decreased blood memory T_{FH} cells, together with a severe deficiency of memory B cells, so that CVID patients are more susceptible to various bacterial, viral and fungal infections⁹⁷.

Multiple mouse models show that B cells play a fundamental role in the generation of GC T_{FH} cells by interacting with T_{FH} precursors¹⁴. This is also the case in humans, as patients with X-linked agammaglobulinemia, who lack mature B cells because of a deficiency of the tyrosine kinase Btk, and those with CVID, who have a significantly reduced number of B cells (<2% in lymphocytes), display significantly reduced frequencies of blood memory T_{FH} cells⁹⁸. Patients with hyper-IgM syndrome (HIGM) caused by mutations in either functional CD40L or CD40, show severely impaired GC formation, as well as reduced blood memory T_{FH} cells and memory B cells, excess IgM production, multiple recurrent infections, which indicates the importance of CD40-CD40L interactions for GC and T_{FH} development in humans^{82, 99, 100}. SAP-deficient mice show profoundly altered GC reactions and generation of T_{FH} cells¹⁰¹, as SAP is required for durable interactions between helper T cells and B cells. Consistently, patients with X-linked lymphoproliferative disease (XLP), caused by SAP deficiency, show impaired formation of GCs

and impaired development of memory B cells¹⁰², susceptibility to EBV infection, and the development of lymphoma¹⁰³. Interestingly, both SAP-deficient mice and human patients with X-linked lymphoproliferative disease show normal frequencies of blood memory T_{FH} cells⁹⁴, suggesting that at least a subset of circulating blood T_{FH} cells develop before GC T_{FH} formation.

Mutations in the intracellular signaling molecule STAT3 cause most cases of hyper IgE syndrome. People with this syndrome are susceptible to a narrow spectrum of infections linked to defective T_{H17} responses and show altered antibody responses with reduced blood memory T_{FH} cells^{61, 104}, suggesting the important role of STAT3 in T_{FH} cells generation in humans. The reduced number of blood memory T_{FH} cells in STAT3-mutant subjects might be dependent on both B cell-intrinsic and T cell-intrinsic defects. Notably, upon exposure to IL-12, human STAT3 mutant naive CD4⁺ T cells are induced to express high amounts of several T_{FH} molecules, such as CXCR5, ICOS and BCL6, despite lower expression of IL-21⁶¹. Thus, other molecules such as STAT4 might functionally compensate for the lack of STAT3.

1.5.2 T_{FH} cells and autoimmunity

The generation of autoantibodies is a hallmark of autoimmune disease. Autoantibodies target a broad range of self-antigens (nuclear components, organ specific antigens and soluble factors) and can profoundly dysregulate the function of multiple organs or systems through a variety of mechanisms. Autoantibodies result from a breakdown of tolerance mechanisms during B cell development¹⁰⁵, from T cell-dependent or -independent B cell activation, or as a consequence of somatic mutation and rogue selection within GCs¹⁰⁶. There's now evidence in mice and in humans that aberrant generation and/or activation of T_{FH} cells and extrafollicular helper T cells contributes to the pathogenesis of autoimmune disease.

In patients with systemic lupus erythematosus (SLE), was suggested increased GC response that caused an increased frequency of CD27⁺CD38^{hi} somatically mutated antibody-producing plasmablasts in peripheral blood^{107, 108}. Because SLE patients display higher frequencies of self-reactive mature naive B cells than healthy controls¹⁰⁹, SLE patients seem predisposed to the development of a broader range of autoantibodies than healthy subjects. Early studies showed increases in the frequency of CD4⁺ICOS⁺ T cells in the peripheral blood of patients with SLE¹¹⁰ and in the frequency of CD4⁺CXCR5⁺ T cells in the peripheral blood of patients with myasthenia gravis¹¹¹. Furthermore, in myasthenia gravis, the frequency of CD4⁺CXCR5⁺ T cells in the circulation positively correlated with clinical disease scores^{111, 112}. Subsequent studies reported increases in circulating CD4⁺CXCR5⁺ICOS^{hi}PD-1^{hi} T cells in some patients with SLE or Sjögren's syndrome, and these were shown

to correlate with autoantibody titres, frequencies of circulating GC B cells, plasma cells and disease severity^{113, 114}. Increased frequencies of circulating CXCR5⁺ICOS^{hi} or CD4⁺CXCR5⁺PD-1⁺ T cells have also been observed in patients with rheumatoid arthritis and autoimmune thyroid diseases (Graves' disease and Hashimoto's thyroiditis), and have been shown to be accompanied by elevated levels of serum autoantibodies^{115, 116}(Table 3). Accumulating evidence also indicates that patients with autoimmune disease display an alteration in the balance of blood memory T_{FH1}, T_{FH2} and T_{FH17} cells. In patients with juvenile dermatomyositis, adult SLE and Sjögren's syndrome, T_{FH1} cells are underrepresented among blood memory T_{FH} cells, whereas T_{FH2} and/or T_{FH17} cells are overrepresented. Such alterations were found to correlate with disease activity, serum antibody and the frequency of blood plasmablast^{93, 117}.

These observations suggest that patients with active autoimmune disease display aberrant T_{FH} responses; this can be monitored through assessment of the increase of activated blood memory T_{FH2} and T_{FH17} cell subsets, and decrease of T_{FH1} cell subset. This indicates that T_{FH} cells are likely to contribute to disease pathogenesis, and it highlights the possibility of targeting T_{FH} cells to effectively treat antibody-mediated autoimmune conditions.

1.5.3 T_{FH} cells in cancer

T_{FH} cells play important roles in supporting the growth and survival of follicular B cell tumors. Evidence for a pathogenic role of T_{FH} cells in B cell lymphoma first came from studies demonstrating that the number of T cells infiltrating B cell tumors was an important predictor of outcome¹²⁷. Furthermore, T_{FH} cells themselves can give rise to a peripheral T cell tumor known as angioimmunoblastic T cell lymphoma (AITL)¹²². Other peripheral T cell tumors also present with phenotypic features and genetic abnormalities that suggest a T_{FH} origin. AITL, which accounts for ~20% of peripheral T cell lymphomas (PTCLs), is an aggressive tumor associated with a poor survival rate. The neoplastic T cells account for only a small fraction of the lymphoid infiltrate and are mixed with a large number of reactive immune cell types. Genetic profiling of AITL and immunohistochemical analysis of neoplastic T cells have provided strong evidence that T_{FH} cells are the normal cellular counterpart of the neoplastic cells in AITL, which typically express BCL6, CD10 and other T_{FH} markers, including CXCL13 and PD-1¹²³. Mutations in *TET2*, *IDH2*, *DNMT3A* and *RHOA* are commonly found in AITL^{124, 125}. Although the first three genes are known to play a role in DNA methylation and epigenetic modification of gene expression, how these mutations selectively affect T_{FH} cells remains to be understood. Up to 40% of other PTCLs (PTCLs-not otherwise specified (NOS)) also express T_{FH} markers, and some share genetic mutations typically found in AITL, including in *TET2*, *IDH2* and *RHOA*. For this reason, these tumors are now being referred to as T_{FH}-like PTCL-NOS¹²⁶. Some of the cardinal features of AITL and FTCL include B cell

activation, follicular hyperplasia, hypergammaglobulinaemia and autoantibody production. These humoral immunity-associated features of AITL and FTCL probably reflect the dysregulated activity of malignant T_{FH} cells, which parallels the aberrant function of T_{FH} cells in autoimmunity.

Recently, T_{FH} cells were found to infiltrate solid-organ tumors, where they might play both protective and pathogenic roles. A high proportion and altered distribution of T_{FH} cells has been reported in colorectal cancer¹²⁸ and non-small cell lung cancer¹²⁹ and T_{FH} cells infiltrate thymomas, correlating with the severity of myasthenia gravis¹²⁶. Moreover T_{FH} cells are a component of the inflammatory infiltrate of breast cancer, and their presence was associated with increased survival¹³⁰. Analysis study on various immune cells isolated from human colorectal tumors revealed that the infiltration of B cells and T_{FH} cells, which is associated with the expression of *CXCL13* and *IL21*, positively correlates with patient survival.

Table 3: Human diseases associated with aberrant T_{FH} cell function²

Human disease	T _{FH} cell-related phenotype	Correlated pathology
Autoimmunity		
Systemic lupus erythematosus	- Increased frequencies of circulating CD4⁺CXCR5⁺PD1^{hi} cells, CXCR5⁺ICOS^{hi} cells or ICOS^{hi} T cells - Increased serum levels of IL-21 and CXCL13	- Increased severity of end-organ damage - Higher serum levels of dsDNA-specific autoantibodies
Sjögren's syndrome	Increased frequencies of circulating CD4⁺PD1^{hi} T cells, CD4⁺CXCR5⁺ICOS^{hi} T cells or CD4⁺CXCR5⁺CCR6⁺ (T_H17-type) T cells	Higher serum levels of autoantibodies
Rheumatoid arthritis	- Increased frequencies of circulating CD4⁺CXCR5⁺PD1^{hi} cells or CD4⁺CXCR5⁺ICOS^{hi} T cells - Increased serum levels of IL-21	- Higher serum levels of CCP-specific autoantibodies - Higher disease score and serum levels of IL-21- and CCP-specific autoantibodies
Juvenile dermatomyositis	Increased T_H2- and T_H17-like subsets among circulating CD4⁺CD45RO⁺CXCR5⁺ T cells	- Higher disease score - Increased number of circulating plasmablasts
Autoimmune thyroid disease (Graves' disease or Hashimoto's thyroiditis)	Increased frequencies of circulating CD4⁺CXCR5⁺PD1^{hi} T cells or CD4⁺CXCR5⁺ICOS^{hi} T cells	Increased titres of serum autoantibodies against thyroid stimulating hormone receptor and thyroglobulin
Myasthenia gravis	Increased frequencies of circulating CD4⁺CXCR5⁺ T cells or CXCR5⁺CD57⁺ T cells	Increased disease severity
Immunodeficiency		
X-linked lymphoproliferative disease	Normal frequency of circulating CD4⁺CXCR5⁺ T cells but impaired helper function in vitro	Impaired cytokine production, ICOS expression and B cell help in vitro
CVID (ICOS deficiency) and hyper-IgM syndrome (CD40 or CD40L deficiency)	Reduced frequency of circulating CD4⁺CD45RO⁺CXCR5⁺ T cells	Impaired development of T_{FH} cells owing to an inability to form GCs
AD-HIES (STAT3 deficiency)	- Reduced frequency of circulating CD4⁺CD45RO⁺CXCR5⁺ T cells - Impaired generation of T_{FH}-like cells from naive precursors in vitro	Inability of naive CD4⁺ T cells to express IL-21 and to provide help to B cells in response to T_{FH} cell-inducing cytokines (for example, IL-12)
HIV	- Increased frequency of T_{FH} cells in lymph nodes of HIV-infected individuals - Enrichment of HIV-specific CD4⁺ T cells in the T_{FH} cell compartment	Positive correlations with plasma viraemia, numbers of GC B cells and plasma cells, hypergammaglobulinaemia and virus-specific antibodies
Lymphoma		
AITL and FTCL	- Increased number of CXCR5⁺PD1^{hi}ICOS^{hi}OX40⁺SAP⁺BCL-6⁺MAF⁺ malignant cells - Detectable expression of CXCL13 and IL-21	Might be correlated with aberrant humoral features such as follicular hyperplasia, hypergammaglobulinaemia and autoantibody production in these PTCLs

AD-HIES, autosomal dominant hyper-IgE syndrome; AITL, angioimmunoblastic T cell lymphoma; BCL6, B cell lymphoma 6; CCP, cyclic citrullinated peptide; CCR6, CC-chemokine receptor 6; CD40L, CD40 ligand; CVID, common variable immunodeficiency syndrome; CXCL13, CXC-chemokine ligand 13; CXCR5, CXC-chemokine receptor 5; dsDNA, double-stranded DNA; FTCL, follicular T cell lymphoma; GC, germinal centre; ICOS, inducible T cell co-stimulator; IL, interleukin; PD-1, programmed cell death protein 1; PTCL: peripheral T cell lymphoma; SAP, SLAM-associated protein; STAT3, signal transducer and activator of transcription 3; T_{FH}, T follicular helper; TH, T helper.

1.6 microRNAs: differentiation and mechanisms of action

Recent high-throughput transcriptomic analysis have revealed that eukaryotic genomes transcribe up to 90% of genomic DNA¹³¹. Only 1-2% of these transcripts encode for proteins, whereas the vast majority are transcribed as non-coding RNAs (ncRNAs). Evolutionary, the repertoire of protein-coding genes has remained relatively static, whereas the amount of non-coding sequences has markedly increase with the complexity of the organism¹³². This point together with the fact that most putative ncRNAs are expressed at substantially lower levels than messenger RNAs (mRNAs), suggests that these ncRNAs have regulatory functions. Classification of these ncRNAs by length leads to divide ncRNAs in two main categories: small and long ncRNAs. In the first group there are microRNAs (18-25 nt), small-interfering RNAs (19-23 nt), piwi-interacting RNAs (26-30 nt), small nuclear and nucleolar RNAs (60-300 nt), promoter-associated small RNAs (20-200 nt), centromer repeat-associated small interacting RNAs¹³³.

MicroRNAs (miRNAs) represent so far the best characterized class of ncRNAs in terms of both expression and function. miRNAs are highly conserved non-coding single-stranded small RNA molecules that control gene expression post-transcriptionally by binding mainly the 3' untranslated region (UTR) of target mRNA¹³⁴. This results in the degradation of target mRNA (through deadenylation) or inhibition of its translation¹³⁴. miRNA genes are transcribed into primary miRNAs (pri-miRNAs) by RNA polymerase II or rarely by RNA polymerase III. The pri-miRNA is next endonucleolytically cleaved by the nuclear microprocessor complex formed by the RNase III enzyme Drosha and

the DiGeorge critical region 8 (DGCR8) protein into hairpin structures of about 60 nucleotides known as precursor miRNAs (pre-miRNAs). Exportin 5 shuttles pre-miRNAs from the nucleus into the cytoplasm where the RNase III Dicer cleaves off the hairpin loop of the pre-miRNA. The resulting duplex of 22 nucleotides segregates and the mature single-stranded miRNA associates with Argonaute (AGO) proteins and other accessory proteins to form the miRNA-induced silencing complex (miRISC), which mediates the translational repression and the increased degradation of its mRNA targets. The core components of the mammalian miRISC consists of one of four Argonaute proteins (AGO1–4) and Argonaute-bound glycine-tryptophan 182 (GW182) proteins, which in mammals are mainly three, TNRC6A –B and –C. The most important AGO protein is AGO2, that has an enzymatically competent RNaseH-like Piwi domain, which cleaves mRNA at the center of the siRNA-mRNA duplex, but also AGO1 and AGO4 are involved in the miRNA mediated mRNA repression^{135, 136}. A mature miRNA bound to an AGO protein forms the core of the miRISC and the AGO protein recruits other protein complexes that antagonize translation and modulate the expression of the targeted mRNA (Fig. 13)^{135, 136}. miRISC represses gene expression post-transcriptionally through two main mechanisms: the repression of translation and the mRNA decay. The miRNA-mediated translational repression happens because miRISC complex can inhibit translation initiation by interfering with eukaryotic translation initiation factor 4F (eIF4F)-cap recognition and 40s small ribosomal recruitment or can antagonizing 60s subunit joining and preventing 80s ribosomal complex formation. Indeed

miRISC can inhibit translation at postinitiation steps by inhibiting ribosome elongation, inducing ribosome drop-off or facilitating protolysis of nascent polypeptide (Fig 14a). Moreover, the interaction of GW182 protein with poly(A)-binding protein (PABP) interferes with PABP-eIF4G association and mRNA circularization, contributing to repression of translation initiation¹³⁶. For mRNA decay, GW182 recruits the carbon catabolite repression 4 protein-negative on TATA-less protein (CCR4-NOT1) and poly(A) nuclease 2 and 3 (PAN2-PAN3) deadenylase complex to facilitate deadenylation of the poly(A) tail. Following deadenylation, the 5-terminal cap (m⁷G) is removed by the decapping the DCP1–DCP2 complex, and mRNA decay is effected by the Xrn1 5'–3' exonuclease¹³⁶ (Fig. 14b).

Therefore, the effect of miRNA-mediated repression can be observed at both the protein and the mRNA level. The miRNA provides specificity through Watson-Crick complementary base-pairing with target mRNAs. Generally in mammals, miRNAs base pair with their targets imperfectly but perfect base-pairing occurs between nucleotides in positions 2–8 from the 5' end of a miRNA, termed the “seed sequence”, and 6-8 nucleotides within the 3' UTR of the target mRNA, called the “miRNAs recognition element” (MRE): these are major determinants of target recognition. In addition an A residue across position 1 of the miRNA and A or U across position 9 improve miRNA activity¹³⁶. Generally, miRNA-mRNA duplexes contain mismatches and bulges in the central region (miRNA positions 10–12) that prevent endonucleolytic cleavage of mRNA by an RNAi mechanism¹³⁵. Complementarity in the 3' half of the miRNA can

contribute to binding and “seedless” targets that rely on non-seed sequences for binding also occur. Most functional miRNA-binding sites occur in the 3′ untranslated region (UTRs) of target mRNAs and many of these are highly conserved, which indicates that miRNAs have co-evolved with their targets. Anyway miRNAs may also target 5′UTR and coding regions of target mRNA¹³⁶. Target genes are frequently inhibited by combination of several miRNAs and similarly each miRNA can target many genes at the same time. These principles have been exploited to develop algorithms for the bioinformatic prediction of miRNA targets¹³⁷. Although these algorithms are useful for hypothesis generation, they remain imperfect; any predicted targets must be experimentally confirmed and many true targets are missed.

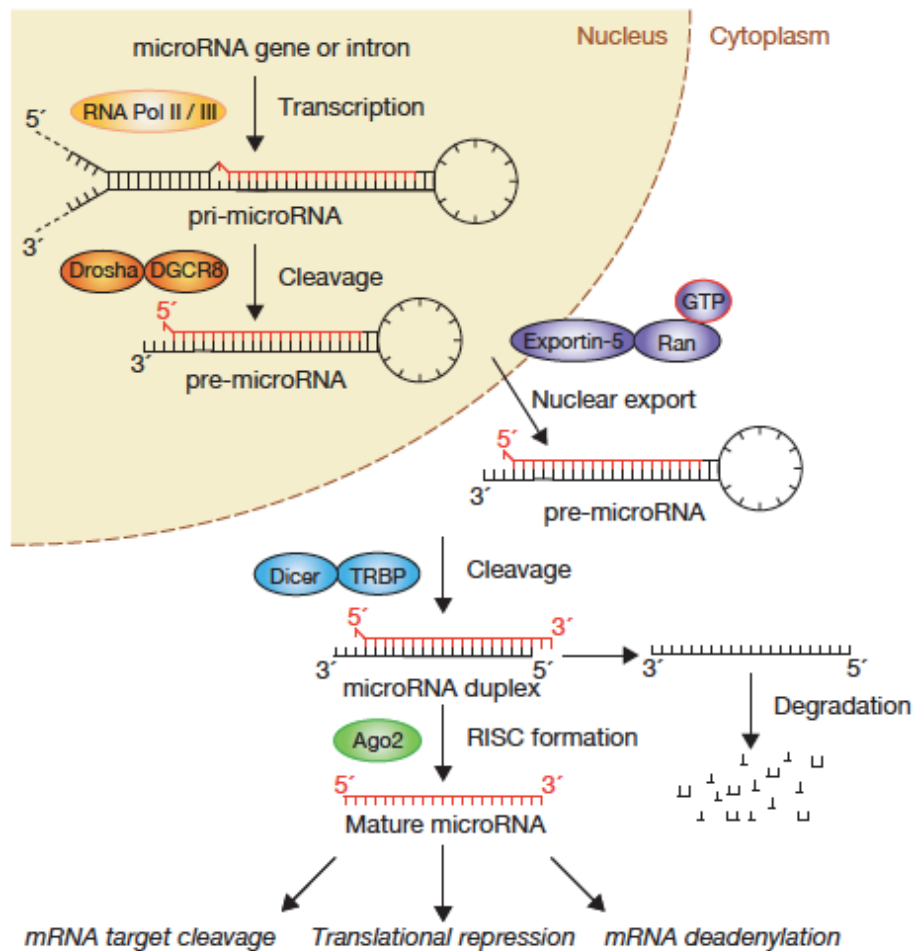


Figure 13: The canonical pathway of microRNA processing¹³⁸. The miRNA processing pathway has long been viewed as linear and universal to all mammalian miRNAs. This canonical maturation includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 in the nucleus. The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP. In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational

repression or deadenylation, whereas the passenger strand (black) is degraded. In this review we discuss the many branches, crossroads and detours in miRNA processing that lead to the conclusion that many different ways exist to generate a mature miRNA.

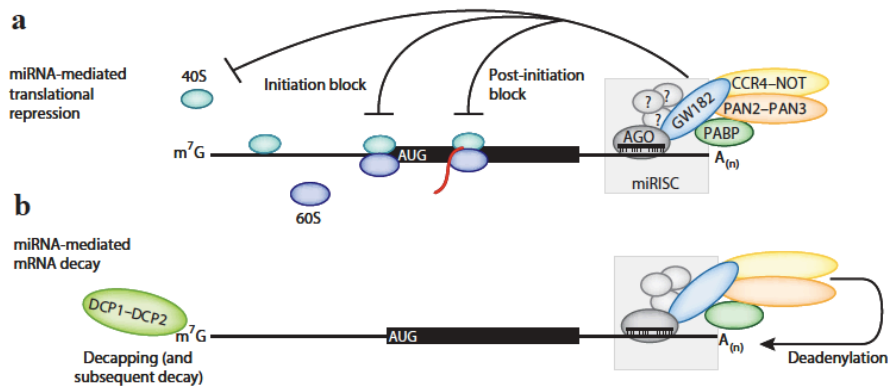


Figure 14: miRISC-mediated gene silencing¹³⁶. See text for details. AGO: argonaute protein; PABP: poly(A)-binding protein; CCR4: carbon catabolite repression 4 protein; NOT1: negative on TATA-less protein; PAN2-PAN3: poly(A)-nuclease 2-3.

1.6.1 microRNA's role in lymphocytes cells biology

Expression of miRNA is tissue specific, and changes in miRNA expression in a tissue have been associated with various human diseases, including cancer, inflammation and chronic viral infection.

It has now become clear that a complex network of transcription factors, epigenetic changes and post-transcriptional regulators is responsible for the development and the maintenance of the different T_H cell subsets and their characteristic gene expression programmes^{137, 139}. Also miRNAs are important modulators of T_H cell fate decisions, plasticity and effector functions. Their fine-tuning activity is particularly well suited to their function early in the response when small changes in the expression of key genes can have a big effect on cell fate. Cell-specific deletion of essential factors in the miRNA biogenesis pathway, such as Dicer¹⁴⁰, Drosha¹⁴¹ and the microprocessor complex subunit DGCR8, decreased the survival and the proliferation of T cells following activation.

Effector T_H cell differentiation is highly influenced by the strength of TCR stimulation, the nature of the co-stimulatory molecules expressed on antigen-presenting cells and the cytokine microenvironment¹⁴². Among the miRNAs that are substantially downregulated after T cell activation, some, such as miR-29, inhibit the differentiation of particular T_H cell subsets¹⁴³, others, such as miR-125, seem to inhibit effector T cell differentiation in general. miR-125b is highly expressed in human naive CD4⁺ T cells compared with various memory T cell populations, and it inhibits the expression of several

genes that are involved in T cell differentiation, including *IFNG*, *IL2RB*, *IL10RA* and *PRDMI* (which encodes BLIMP1)¹⁴⁴.

During their development in the thymus, T cells undergo positive selection and negative selection. In the periphery, the activation of mature naive T cells that encounter foreign antigens leads to clonal selection and differentiation into effector T cells. In all of these thymic and peripheral processes, the sensitivity of a TCR to recognize cognate peptides has important implications for the fate of the T cell. The signalling cascade downstream of TCR activation is then highly regulated at multiple levels, and several miRNAs have been shown to be important in this regulation.

The miR-181 miRNA family is one such regulator of TCR signalling that has clear effects on T cell development and homeostasis. The inhibition of miR-181 in thymocytes decreases TCR sensitivity and impairs positive and negative selection in the thymus¹⁴⁵.

miR-146a is an important feedback regulator of nuclear factor- κ B (NF- κ B) signalling. It is abundantly expressed in human memory T cells. miR-146a targets TNF receptor-associated factor 6 (*Traf6*) and IL-1 receptor-associated kinase 1 (*Irak1*), which provides a negative feedback loop that controls the intensity and the duration of NF- κ B signalling¹⁴⁶. miR-146a deficiency in T cells causes hyper-responsiveness to TCR signals and unresolved T cell-mediated inflammation¹⁴⁶.

The miR-17~92 cluster comprises six miRNAs from four miRNA families that regulate several aspects of T cell activation as well as the subsequent fate of the activated T cells. The overexpression of miR-17~92 in T cells causes lymphoproliferation and an autoimmune-

like disease that is characterized by increased serum antibody levels and an increased production of autoantibodies¹⁴⁷. These miRNAs mediate their effects through many direct target genes, including PTEN and the pro-apoptotic BCL-2-interacting mediator of cell death (*Bim*)^{147, 148}.

miR-155 is the miRNA that is most highly induced by TCR stimulation^{149, 150} and it targets a phosphatidylinositol phosphatase, SH2 domain-containing inositol polyphosphate 5' phosphatase 1 (*Shp1*)^{148, 151}. miR-155 deficiency decreases T cell-dependent humoral and cellular immune responses, probably as a result of the absence of its activity on several mRNA targets^{150, 152}.

IL-2 is an important regulator of CD4⁺ T cell metabolism and proliferation¹⁵³. It is produced by activated T cells and can function in an autocrine manner. miR-146a has been suggested to be a negative regulator of IL-2 production in activated human CD4⁺ T cells, and miR-9 has been described as a positive regulator of IL-2 production, possibly through the direct targeting of *PRDM1*^{154, 155}. In addition, miR-182 enhances clonal selection downstream of IL-2 receptor signalling by inhibiting forkhead box protein O1 (FOXO1), which is a transcription factor that is highly expressed in naive T cells and that limits T cell proliferation¹⁵⁶.

MicroRNAs play essential roles also in several aspects of B cell development and immune function, including the GC response¹⁵⁷. Various human and murine B cell subsets have been profiled by microarrays and sequencing, resulting in a comprehensive assessment of miRNA expression in these cells^{158, 159}. The specific functional roles of some of these miRNAs have been elucidated.

miR-155 is highly expressed in GC B cells, and miR-155-deficient and miR-155-overexpressing mice revealed important roles for this miRNA in several aspects of the GC response, including GC size and number, antibody production, class-switch, affinity maturation, and cytokine production by T_H cells^{150, 160}. miR-155 negatively regulates the generation of class-switched IgG⁺ plasma cells in part through inhibition of the transcription factor Pu.1¹⁶¹. Deletion of miR-155 in lupus-prone Fas^{lpr} mice alleviated the systemic autoimmune response by decreasing the levels of autoantibodies in these mice. AID is another important direct target of miR-155. Since miR-155 promotes the GC B cell response, it is interesting that at the same time it inhibits AID, which is required for somatic hypermutation and also for class-switch recombination. Studies in mice demonstrated that Bcl6 inhibits the expression of several miRNAs, including miR-155, suggesting that coordinated spatio-temporal regulation of Bcl6 and miR-155 expression within the GC may be important for the generation of high-affinity B cells. Several other miRNAs have been implicated to be important regulators of the GC B cell response^{36, 162}.

miR-185 controls B cell activation through inhibition of Bruton tyrosine kinase leading to dampened BCR signaling. Like miR-155, miR-181b also impairs class-switch recombination in activated B cells by targeting AID. miR-150 is highly expressed in naive B cells and limits the magnitude of the GC response. In contrast, miR-125b overexpression promotes GC B cell differentiation by inhibiting genes that drive plasma cell differentiation, including Blimp-1 and IRF4.

In summary, miRNAs can have powerful effects on biological processes by regulating genes that control signalling thresholds. Several miRNAs function at multiple levels to regulate the development, the homeostasis and the activation of T and B cells. These effects have important consequences for T and B cell function, and they are complemented by the miRNA-mediated regulation of particular lymphocytes differentiation pathways and effector mechanisms.

1.6.2 Regulation of T_{FH} cells differentiation by miRNAs

Several miRNAs are differentially expressed in T_{FH} cells compared with other T_H cell subsets¹⁵⁸. miRNAs are essential for an early stage in T_{FH} cell differentiation, as a adoptively transferred *DGCR8*-deficient CD4⁺ T cells fail to upregulate CXCR5 and downregulate CC-chemokine receptor 7 (CCR7), and this prevents their migration to the T cell–B cell border and into B cell follicles, leading to a failure in the generation of CXCR5⁺Bcl6⁺ T_{FH} cells upon immunization with a protein antigen¹⁶³. This defect is in sharp contrast to the role of miRNAs in other T_H cell subsets, such as in T_{H1} and T_{H2} cells, as these cells can still be generated from naive miRNA-deficient CD4⁺ T cells, even with aberrantly high efficiency in some cases^{140, 143}. The fact that T_{FH} cell differentiation strictly requires a transcriptional repressor (BCL6) and post-transcriptional repressors (miRNAs) indicates that the silencing of alternative differentiation pathways has a crucial role in specifying or maintaining the T_{FH} cell gene expression programme. Only a few studies have provided

insights into the roles of individual miRNAs or miRNA families in T_{FH} cells, and these studies have been performed mainly in mouse cells.

The miR-17~92 cluster promotes T_{FH} cell differentiation and function¹⁶³; in a recent study *Baumjohann et al.*¹⁶³ showed that miR-17~92 cluster contributed to the differentiation of an early CXCR5^{hi}Bcl6^{hi} T_{FH} cell population in part by targeting the tumor suppressor *PTEN*¹⁶³. In a viral infection model, miR-17~92 also prevented the expression of T_{FH} cell-inappropriate genes that are characteristic of T_{H17} or T_{H22} cells, including *Ccr6*, *Il22* and *Rora*, which encodes the transcription factor RAR-related orphan receptor alpha (RORα)¹⁶³. Derepression of the miR-17~92 target gene PH domain leucine-rich repeat-containing protein phosphatase 2 (*PHLPP2*), which is an AKT phosphatase involved in the ICOS-PI(3)K-signalling pathway, has been linked to the defective migration of activated miR-17~92-deficient T cells into B cell follicles¹⁶⁴. These findings highlight the ability of miRNAs to ensure the fidelity of T_{FH} cell subset-specific gene expression programme and to modulate the expression of targets that are important for T_{FH} cells differentiation.

In a recent study *Pratama et al.*¹⁶⁵ found that miR-146a is highly expressed in mouse and in human T_{FH} cells and that in mouse miR-146a profoundly represses T_{FH} cells numbers: in its absence there is a spontaneous expansion of T_{FH} and GC B cells that precedes myeloid cell dysregulation and this is not as consequence of T_{reg}-cell functional deficiency. miR-146a can lead to repression of multiple T_{FH} cells-expressed mRNAs, including *Icos*, *Slamf1*, *Slamf6*, *Cd84*, *Stat1*, *Cxcr4* and *Notch1*. *Icos* was the most robustly and autonomously

upregulated miR-146a target across T cells of miR-146a-deficient mice. So this miRNA is able to repress Icos signalling, regulating T_{FH} and B cells accumulation and maintaining immunological tolerance¹⁶⁵. In this way it has an opposite role compared to miR-17~92 cluster, that was shown to promote T_{FH} cells differentiation.

miR-10a inhibits the conversion of peripherally derived T_{reg} cells to T_{FH} cells under lymphopenic conditions by directly targeting *Bcl6* and its co-repressor *Ncor2*¹⁶⁶.

Further work is needed to comprehensively profile miRNA expression in developing T_{FH} cells and to determine how these miRNAs contribute to the balance between T_{FH} cell and effector T cell differentiation, as well as to the maintenance of T_{FH} cell identity. Furthermore, because of microRNAs can have different expression profiles and function among species, such as human and mouse¹⁴⁴, further studies have to be performed in order to understand the roles of microRNA in human T_{FH} cells differentiation and function.

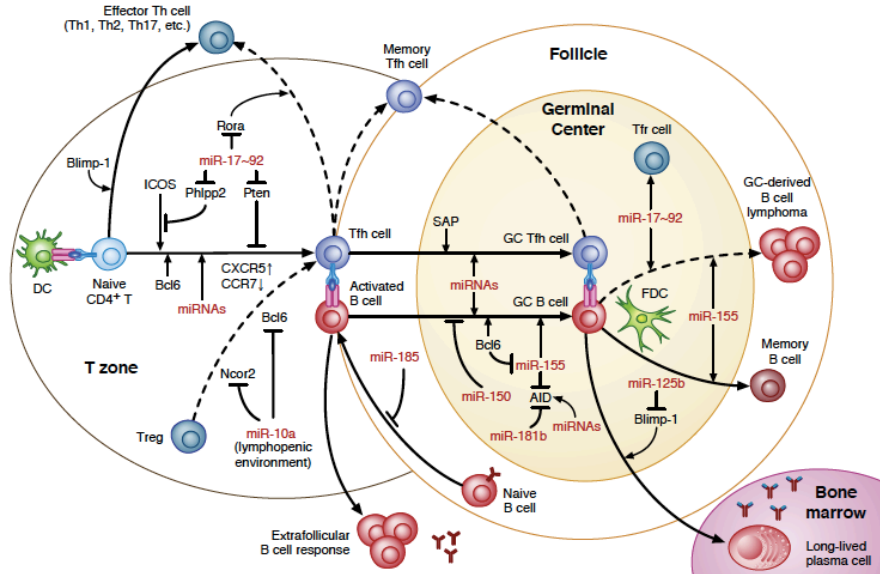


Figure 15: MicroRNAs regulation of the germinal center response¹⁶⁹: The induction of the T_{FH} cell gene expression program is dependent on miRNAs expression by T cells. miR-17~92 promotes T_{FH} cell differentiation by repressing *PTEN* and *PHLPP2*, the latter being an important regulator downstream in the ICOS signaling pathway. In addition, miR-17~92 prevents co-expression of effector T_H cell genes that are normally not expressed by T_{FH} cells through repression of *RORA*. The generation of T follicular regulatory (T_{FR}) cells residing in the GC is particularly sensitive to miR-17~92 dosage. miR-10a prevents the conversion of FOXP3⁺ T_{reg} cells into T_{FH} cells under lymphopenic conditions. The T_{FH} gene expression program may serve as an intermediate state in effector T_H cell subset differentiation, and T_{FH} cells may also persist as memory cells. While most activated B cells enter the extrafollicular pathway, some clones, together with T_{FH} cells, reenter the follicle in a process that requires BCL6 and SLAM-associated protein (SAP) expression by B and T cells, respectively, to establish GCs.

1.7 AIM OF THE THESIS

In this thesis we investigated miRNAs expression patterns in human T_{FH} and $CD4^+$ naive T cells from secondary lymphoid organs (SLO), defining a T_{FH} -specific miRNA signature. Among differentially expressed miRNAs in T_{FH} versus $CD4^+$ naive T cells we focused on hsa-miR-31-5p (miR-31), that is strongly down-regulated in human T_{FH} cells in comparison with other $CD4^+$ T cell subsets, and that specifically modulates the expression of molecules that are relevant for T_{FH} functionality. We also demonstrated that the expression of miR-31 in T_{FH} cells is regulated by the T_{FH} master transcription factor BCL6. For the first time we provided evidences of the role of miRNAs in regulating molecular pathways that control human T_{FH} cells differentiation and function and in turn are able to modulate human humoral immunity.

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2. microRNAs role in human follicular helper T cells differentiation and function

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Abstract

Follicular helper T cells (T_{FH}) are a separate $CD4^+$ T helper subset specialized in provision of help to B cells. They develop independently from other T cell subsets and are critical for humoral immunity, including the generation of long-lived and high affinity plasma cells and memory B cells. Despite their crucial role, the molecular mechanisms underlying human T_{FH} cells differentiation are still poorly understood. MicroRNAs (miRNAs) are highly conserved non-coding single-stranded small RNA molecules that control gene expression post-transcriptionally by binding the 3' untranslated region of target mRNA. To investigate the role of miRNAs in human T_{FH} cells biology we performed a broad analysis of miRNA expression on T_{FH} and naive $CD4^+$ T cells sorted from human adenoids and identified a T_{FH} -specific miRNAs signature composed by 17 miRNAs. Among them, we showed that hsa-miR-31-5p (miR-31) is specifically and strongly down-regulated in human T_{FH} cells in comparison with $CD4^+$ naive T cells from secondary lymphoid organs and with other T cells subsets from peripheral blood. We found that miR-31 regulates the expression of BTLA, SAP and CD40L, three molecules crucial for T_{FH} activity. Importantly, over-expression of miR-31 in primary human T_{FH} cells severely impaires their ability to provide help to B cells. Finally, we showed that the B cell lymphoma 6 protein (BCL6), the master transcription factor of T_{FH} cells, regulates the expression of miR-31. Our results demonstrate a key role of miR-31 in human T_{FH} cells biology and function and elucidate the molecular mechanism that correlates expression of the transcriptional repressor BCL6 with upregulation of molecules defining T_{FH} phenotype and function.

Introduction

Germinal Centers (GCs) are specialized structures in B cell follicles of secondary lymphoid tissues, where somatic hypermutation of immunoglobulin (Ig) variable region genes and selection of high-affinity B cells occur, followed by differentiation of long lived memory cells or plasma cells (PC). This process ensures the development of long-lived humoral immunity after infection or vaccination with T cell-dependent (TD) antigens¹. In the last decade, T follicular helper (T_{FH}) cells have emerged as the key cell type required for the formation of GCs and the generation of long-lived serological memory¹⁻⁷. T_{FH} cells were firstly described as a subset of CD4⁺ T cells present in human lymphoid tissues characterized by the expression of high levels of the C-X-C chemokine receptor type 5 (CXCR5), the receptor for B-cell follicle produced C-X-C motif chemokine 13 (CXCL13), that allows them to relocate from the T cell zone to the B cell follicles where they primarily provide help to B cells⁸. T_{FH} cells can be distinguished from other CD4⁺ T cell subsets by their low expression levels of cytokines (IFN- γ , IL-4, and IL-17) and transcription factors (T-bet, GATA-3, and ROR γ t) characteristic of T_{H1}, T_{H2}, and T_{H17} cells, respectively. They also express a unique combination of effector molecules that are critical for their development and function, including high levels of the surface receptors ICOS, CD40 ligand (CD40L), OX40, PD-1, BTLA and CD84, the cytokine IL-21 and the cytoplasmic adaptor protein SLAM-associated protein (SAP)¹.

In 2009, the transcriptional repressor B cell lymphoma 6 (BCL6) has been defined as master transcription factor of T_{FH}⁹⁻¹¹. BCL6 function had been mostly associated with B cell fate: within the B lineage, BCL6 expression is largely confined to GC B cells^{12, 13}. BCL6 represses genes that function in differentiation, inflammation, apoptosis and cell cycle control¹⁴, and up-regulates, indirectly, the expression of genes important for GC reactions¹⁵. In T lymphocytes, BCL6 is selectively expressed by T_{FH} cells^{3, 16} and regulates their development through repression of T_{H1}, T_{H2}, and T_{H17} lineage-specific transcription factors and possibly also by repression of miRNAs that function as negative regulators of T_{FH} differentiation. Despite it is proved that miRNAs are essential at an early stage in T_{FH} cell differentiation, as activated DGCR8-deficient CD4⁺ T cells fail to up-regulate CXCR5¹⁷, very little is known about their role in human T_{FH} differentiation and function.

Here we analyzed miRNAs expression patterns in human T_{FH} and CD4⁺ naive T cells from secondary lymphoid organs (SLO), defining a human T_{FH} specific signature composed by 17 miRNAs. Among them, we show that hsa-miR-31-5p (miR-31) is specifically and strongly down-regulated in human GC T_{FH} cells in comparison with CD4⁺ naive T cells from SLO and with other T cells subsets from peripheral blood (PB). This GC T_{FH} cells-specific miRNA is able to modulate the expression of molecules that are relevant for T_{FH} cells biology, such as BTLA, SAP and CD40L, and this in turn compromises the ability of T_{FH} cells to provide B cell help. Finally, we showed that the expression of miR-31 is regulated by BCL6 that binds and represses the activity of the miR-31 host gene promoter

(MIR31HG), a long non-coding RNA gene in which the miR-31 is located¹⁸.

Results

miR-31 is specifically down-regulated in human GC T_{FH} cells

To identify miRNAs specifically modulated in primary human germinal center follicular helper T cells (GC T_{FH}), we sorted by flow cytometry CD4⁺ naive T cells and GC T_{FH} cells from adenoids obtained from pediatric donors, accordingly to phenotypical markers (Fig. 1a and Table S1). We assessed the expression of 664 miRNAs in these purified lymphocyte subsets by quantitative RT-PCR (TaqMan Low Density Array, TLDA). We performed differential expression analysis to compare miRNAs expression of T_{FH} versus naive CD4⁺ T cells, defining a T_{FH}-specific signature composed by 17 miRNAs that are up or down-regulated in T_{FH} cells with respect to naive T cells (Fig. 1b). Among these 17 differentially expressed miRNAs, the hsa-miR-31-5p (called miR-31 from now on) is strongly down-regulated in T_{FH} cells in comparison with CD4⁺ naive T cells and it shows the highest fold change between T_{FH} and CD4⁺ naive T cells (Supplementary Fig. 1).

We validated data obtained from TLDA by quantitative RT-PCR on a new independent set of donors, sorting GC T_{FH} cells from both adenoids (squares) or tonsils (circles), confirming miR-31 down-regulation in T_{FH} cells (Fig. 1c). To assess the degree of miR-31 conservation between human and mouse, we analyzed expression of

murine miR-31 analog (mmu-miR-31-5p) in T_{FH} and CD4⁺ naive T cells purified from spleens of C57BL/6 mice infused with OT-II thymocytes and challenged with ovalbumin vaccination (sorting strategy, see Table S2). We observed a very low expression of murine miR-31 in both T_{FH} and CD4⁺ naive T cells and an expression pattern that was opposite to that of human cells (supplementary Fig. 2). We finally check the expression of miR-31 in other effector lymphocytes subsets from human SLO (Fig. 1d, black bars) and peripheral blood (Fig. 1d, gray bars), observing that miR-31 expression is lower in T_{FH} cells compared to the other subsets (for sorting strategy, Table 1). Thus, we defined for the first time a human T_{FH} cells miRNA signature and we found that miR-31 is strongly and specifically down-regulated in human T_{FH} cells in comparison with both undifferentiated (CD45RO⁻) CD4⁺ naive T cells and with the other antigen-experienced subsets (CD45RO⁺) from SLO and peripheral blood (PB) as well.

miR-31 directly regulates targets that are relevant in human GC T_{FH} cells biology: *BTLA*, *SH2D1A* and *CD40L*

To identify miR-31 target transcripts, we first performed a transcriptome analysis by RNA sequencing (RNA-seq) on the same CD4⁺ naive T cells and GC T_{FH} cells samples used for the miRNAs analysis. We performed a differential expression analysis of the transcriptome of GC T_{FH} cells versus the one of CD4⁺ naive T cells, using Cufflinks. Among the transcripts that have an expression of at

least 1.5 fold in T_{FH} compared to CD4⁺ naive T cells (Fig. 2a, blue dots) we focused on those transcripts that were predicted targets of miR-31 and whose expression in GC T_{FH} and CD4⁺ naive T cells was inversely correlated to the expression of miR-31 (anti-correlated expressed predicted targets, ACEPTs, of miR-31; see Table S3). We identified 347 ACEPTs (Fig. 2a, red dots) and, among them, we focused on molecules that are important for T_{FH} cell biology and function (Fig. 2a and table S3).

To perform a first screening of those ACEPT transcripts, we took advantage of the dual luciferase assay by cloning their 3' untranslated region (3' UTR) into psiCHECK2 vector down-stream of a Renilla luciferase gene and delivering this construct with the synthetic mature miR-31 (mimic miR-31, Fig. 2b black bars) or a synthetic control miRNA (mimic ctrl, Fig. 2b white bars) in HEK-293T cells. We found that miR-31 regulates the 3' UTRs of CD40 ligand (CD40L), B and T lymphocytes attenuator (BTLA) and SH2D1A (encoding for SAP) (Fig. 2b). Other miR-31 ACEPTs (CD28, CXCR5) were not confirmed as miR-31 targets (Fig. 2b).

To confirm these results in human primary T_{FH} cells, we sorted those cells from SLO and transduced them with lentiviral vectors (LVV) expressing miR-31 (LVV miR-31) or a miRNA scramble (LVV scramble) (Fig. 2c). After 24 hours of transduction we measured levels of CD40L, SAP and BTLA messenger RNAs (mRNAs) by RT-qPCR and observed lower levels of these transcripts in T_{FH} cells treated with LVV miR-31 (black bars) with respect to those treated with the LVV scramble (white bars), confirming in human primary T_{FH} cells what we observed in HEK293T by dual luciferase assay

(Fig. 2d). We also measured the expression of CD40L, SAP and BTLA proteins by cytofluorimetric analysis, observing a statistically significant decrease also at protein levels in cells transduced with LVV miR-31 (Fig. 2e). By these experiments we were able to demonstrate that miR-31 directly regulates the expression of three molecules crucial in T_{FH} biology and function.

Reduced ability of miR-31 transduced T_{FH} to provide help to B cells

Based on our previous findings we asked whether modulation of miR-31 levels in T_{FH} cells might influence their functional activity. To this purpose, we set up a B-helper assay, in which autologous B memory cells were co-cultured with GC T_{FH} cells transduced with LVV miR-31 (blue bars) or LVV scramble (white bars) for 24 hours (Fig. 3a). After 7 days, the IgG or IgM released into the medium by B cells were measured by ELISA and used as a direct measurement of T_{FH} helper activity. We used B cells alone (red bars) or cultured with untreated T_{FH} (grey bars) as negative and positive controls, respectively. B cells co-cultured with LVV miR-31-transduced GC T_{FH} cells (blue bars) produced statistically significant lower amounts of immunoglobulins than the one co-cultured with LVV scramble-transduced GC T_{FH} cells (white bars), meaning that the increased level of miR-31 correlates with the inhibition of the B-helper activity of T_{FH} cells (Fig. 3b). By these experiments, we concluded that beside controlling the phenotype

of T_{FH} cells, miR-31 expression influence also the functional activity of these cells.

BCL6 down-regulates miR-31 expression in human tonsillar naive CD4⁺ T cells

BCL6 has been defined as master transcription factor of T_{FH} and it is essential for CXCR5 expression, T cell migration into follicles and GC formation. *Yu et al.*¹¹ showed that *in vitro* over-expression of BCL6 in CD4⁺ naive T cells from OT-II mice results in up-regulation of typical T_{FH} cells transcripts, such as CXCR5, CXCR4, and PD-1 and down-regulation of CCR7; at the same time it results in more than 50% downregulation of 34 miRNAs out of the 65 that were abundantly expressed¹¹. These data suggest that the transcriptional repressor BCL6 can drive upregulation of typical T_{FH} cells markers through the repression of miRNAs that in turn have these T_{FH} specific molecules as targets. Moreover, *Basso et al.*¹⁵ demonstrated by ChIP for BCL6 followed by quantitative PCR (qChIP) that in mouse GC B cells BCL6 directly targets miR-155 and miR-361 by binding promoter regions and introns of host genes (MIR155HG and CHM respectively) where these miRNAs are located. This BCL6-mediated miRNAs repression determines again an indirect modulation of miR-155 and miR-361 targets that are important for GC reaction, the most important being AICDA¹⁵. Based on these findings we hypothesized that BCL6 could suppress miR-31 expression and this in turn causes the up-regulation of T_{FH} specific molecules that are miR-31 targets

(Fig. 4a). To verify this hypothesis, we sorted CD4⁺ naive T cells from SLO (these cells express BCL6 at very low levels, while miR-31 at high levels in comparison with T_{FH} cells) and transduced them with a LVV encoding for BCL6 (LVV BCL6) or an empty LVV (LVV MOCK) (Fig. 4b). As expected, expression of BCL6 in CD4⁺ naive T cells resulted in the expression of markers characteristic of T_{FH} cells, particularly CXCR5, ICOS and SAP (Fig. 4c, upper graphs). Most importantly, we found that miR-31 levels were significantly reduced in LVV BCL6 transduced cells respect to the LVV mock transduced cells, suggesting that miR-31 expression could be regulated by BCL6 (Fig 4c, lower graph).

In humans, miR-31 is located in the first intron of LOC554202 (miR-31 host gene, MIR31HG). *Augoff et al*¹⁸. recently found a loss of miR-31 expression in Triple-Negative breast cancer (TNBC) cell lines as a direct consequence of hypermethylation of the promoter region of MIRHG, that correlated also with the down-regulation of this lncRNA, suggesting that MIR31HG and miR-31 probably share the same promoter and their expression is co-regulated¹⁸. In accordance with that, available histone modifications data in CD4⁺ naive cells showed an enrichment of H3K4me3 and H3K4me1 characteristic of active promoters (high levels of H3K4me3 and low levels of H3K4me1)¹⁹ at the MIR31HG promoter region and not in the first intron of the gene, suggesting that miR-31 expression is controlled by MIR31HG promoter and not by a different internal promoter. These histone modifications are not observed on T_{FH} cells, indicating that this is not an actively transcribed region in human GC T_{FH} cells

(Supplementary Fig.4, top) in accordance with the different levels of miR-31 observed in this two cell types.

*Hatzi et al.*²⁰ recently performed a BCL6 chromatin immunoprecipitation sequencing (ChIP-seq) on primary human GC T_{FH} cells obtained from healthy donors tonsils. Analyzing these published BCL6 ChIP-seq data, we found an enrichment of BCL6 peaks in a region in the proximity of the transcription starting site (TSS) of MIR31HG. (Supplementary Fig. 4, bottom). These data strengthen our hypothesis that BCL6 can bind and regulate MIR31HG promoter, and in turn miR-31 expression, in GC T_{FH} cells.

To address this issue, we performed *in silico* prediction for BCL6 binding sites (BS) in a region of 5000 bp up-stream the MIR31HG transcription starting sites (TSS) using MatInspector from Genomatix software (Fig. 5a and Table S4). We cloned three different fragments of this region up-stream to the Firefly luciferase gene, in order to perform a Dual Luciferase Assay (Fig. 5b): MIR31HG promoter fragment I contains only a minimal promoter region, while fragment II and fragment III contain one or five predicted BCL6 BS respectively. Finally, to fine-tune the expression of BCL6, we took advantage of the cumate gene-switch inducible expression system²¹ to obtain HEK293T cells stably expressing the CymR repressor (HEKCymR) and BCL6 wild-type (WT), BCL6 deleted of the functional domain that allows the recruitment of other corepressors (BCL6 Δ BTB) or BCL6 deleted of the zinc finger (ZF) DNA binding domain (BCL6 Δ ZF) under a cumate inducible promoter (creating new double-stable cells lines, called HEKCymR CuO_BCL6, HEKCymR CuO_BCL6 Δ BTB or HEKCymR CuO_BCL6 Δ ZF respectively). For Dual

Luciferase assay we transfected HEKCymR cell lines expressing the inducible BCL6 WT or its deleted forms, with a vector containing Firefly Luciferase under the control of the different MIR31HG promoter fragments (fragment I, II, III), together with a plasmid encoding for a Renilla Luciferase as internal normalizer. We added different concentrations of Cumate to the medium to induce proportional BCL6 expression, observing an increase of BCL6 protein expression by FACS proportional to the concentration of the Cumate added (supplementary figure 3c). We observed a decrease in the relative luciferase activity when we used the MIR31HG promoter fragment II and III as we increase the level of BCL6 with Cumate in HEKCymR CuO_BCL6 WT cells (Fig. 5c, red histograms). This reduction is higher when we use fragment III, that contains more BCL6 binding sites than fragment II (Fig. 5c, middle and bottom graphs), and is not observed if we consider the fragment I, containing only the minimal promoter without predicted BCL6 binding site (Fig. 5c, top graph). Moreover, the decrease of relative luciferase activity is not observed or barely detectable when we use HEKCymR_Cuo BCL6 Δ ZF cells or when we use HEKCymR Cuo_BCL6 Δ BTB cells (Fig. 5c, grey and white histograms). These experiments are in line with the recent findings in the literature and demonstrate that BCL6 regulates miR-31 expression by specific modulation of MIR31HG promoter.

Discussion

Since the early 2000s an increasing number of studies better defined T_{FH} cells as an independent $CD4^+$ T cells subset that resides in secondary lymphoid organs, characterized by a specific and unique phenotype ($CD4^+CD45RO^+CXCR5^{+hi}CXCR4^+PD-1^{hi}ICOS^{hi}$), cytokine expression profile ($IL-4^+$, $IL-21^{++}$, $CXCL13^+$ in human), a specific transcriptional profile (BCL6 as master transcription factor, together with MAF, IRF4, BATF and STAT3/STAT4) and with unique functional capabilities¹⁻⁷¹⁻⁷. These cells play a critical and essential role in humoral immunity, thanks to their ability to provide help to B cells for the generation of germinal centers (GC) and with consequent production of high-affinity plasma cells and memory B cells.

Several studies in mice demonstrated that increase or reduction of T_{FH} cells number alters proportionally the GC B cells number, causing either higher plasma cell quantity and increased antibody production⁹ or impaired germinal center B cells, plasma cells and antibody responses²². So developing vaccines regimens that favor T_{FH} cells differentiation might lead to increased antibody responses while developing strategies that control T_{FH} cells number can be useful to limit aberrant B cells responses and the onset of autoimmune diseases. To this aim, we need to better understand the molecular mechanisms regulating human T_{FH} cells differentiation, function and survival. Interestingly, the past few years have seen much progress in the field of miRNA-mediated regulation of lymphocyte development and immune function²³, particularly with respect to $CD4^+$ T helper (T_H)

cells²⁴⁻²⁶. T_H cell differentiation is sensitive to small changes in transcriptional regulation and, since miRNAs have evolved as a mechanism to fine-tune biological processes²⁷, it is probable that miRNAs represent an equally plastic and adjustable system for the regulation of T_H cell differentiation and plasticity. T_{FH} cells in particular are, among all T_H subsets, the only subset in which miRNAs are essential for an early stage cell differentiation²⁸. Up until now, very little is known about miRNAs role in T_{FH} cells and the few studies that define a role of miRNAs in T_{FH} cells have been performed in mouse, defining important roles for miR-17~92 cluster, miR-155 and miR146a in regulating T_{FH} cells differentiation by controlling genes involved in migration and development or by repressing T_{FH} cell subset-inappropriate genes expression^{15, 17, 29-31}.

In our study we characterized for the first time the miRNome of human T_{FH} cells. We performed a differential expression profile of miRNAs expressed in human T_{FH} cells versus CD4⁺ naive T cells isolated from adenoids of pediatric patients and we found a T_{FH}-specific signature composed by 17 differentially expressed miRNAs. Among them, we concentrated on miR-31 that is specifically down-modulated in T_{FH} cells and shows the highest fold change with respect to CD4⁺ naive cells. We showed that miR-31 has the ability to modulate the differentiation of B cells into high affinity plasma cells and memory B cells, by targeting molecules that in T_{FH} cells are essential to maintain stable T-B interaction, to regulate T_{FH} cells function (IL-21 production) and to support B cells survival and differentiation (SAP, BTLA, CD40L respectively)^{1, 32}. miR-31 over-expression in human GC T_{FH} cells using lentiviral vectors severely

impairs their ability to provide help to B cells, causing a decreased secretion of immunoglobulins (IgM and IgG) by syngenic co-cultured memory B cells.

In order to test a possible effect of miR-31 modulation also *in vivo*, we analyzed miR-31 expression in mouse splenic naive and T_{FH} cells. We previously demonstrated that there was relatively good conservation of the overall lymphocyte mouse and human miRNomes (61%) but poor concordance between humans and mice for lymphocyte miRNA signatures³³. Our analysis of mmu-miR-31 showed a very low expression of this miRNA in splenic CD4⁺ naive and T_{FH} cells and an opposite trend of expression in comparison with what observed in human cells (miR-31 expression higher in murine T_{FH} cells than in murine naive CD4⁺ cells). Moreover, mmu-miR-31 is located in exon 3 of lnc-miR-31³⁴, while hsa-miR-31 in an intronic region of lncRNA MIR31HG¹⁸, meaning that they probably have different mechanisms of regulation. There are several other example in literature showing that not all the features of T_{FH} cells in mouse have a counterpart in human T_{FH} cells^{35,36,37,38}. All these evidences outline the importance of studying molecular mechanisms underlying T_{FH} biology in human T_{FH} cells rather than in their murine counterpart, despite the difficulties in obtaining these cells that can be isolated only from secondary lymphoid organs and usually in very low numbers. Our T_{FH} miRNA-signature open the field of investigation of miRNAs role in regulating human humoral immunity and provides suggestion for the therapeutic miRNAs modulation of T_{FH} cells gene networks that can control their proliferation and function.

Giving the fundamental role of miR-31 in human T_{FH} cells, we asked how the expression of this miRNA could be modulated in human T_{FH} cells. The BCL6 transcriptional repressor protein is essential for the formation of both T_{FH} cells and GC B cells; BCL6 deficient mice fail to develop GCs as the result of cell-autonomous effects in each of these cell types^{9,10,11,17}. BCL6 represses critical pathways for GC development including the response to DNA damage, cell activation, and differentiation⁴¹. In GC B and T_{FH} cells several group showed that BCL6 can act as “repressor of the repressors” by targeting miRNAs that in turn repress important GC B and T_{FH} cells molecules. For example BCL6 represses miR-17~92 cluster in mice T_{FH} cells that in turn determines the upregulation of CXCR5 when Bcl6 is over-expressed¹¹. In GC B cells *Basso et al*¹⁵. demonstrated that BCL6 can bind the MIR155HG and CHM promoters, causing the repression of miR-155 and miR-361 expression¹⁴. This in turn causes the up-regulation of AID, that is a miR-155 target¹⁴. Thus the transcriptional repressor BCL6 is able to induce up-regulation of several proteins by inhibiting the expression of other important repressors, but there are no published data supporting this statement in human primary T_{FH} cells. In this work, we have shown that BCL6 represses miR-31 expression possibly by a specific interaction of BCL6 in the promoter region of MIR31HG, a lncRNA in which hsa-miR-31 is located¹⁸. Further more, in a recent study BCL6 ChIP-seq was performed on human tonsil GC B and T_{FH} cells . ChIP-seq data analysis revealed an increase in BCL6 binding density in a region around the TSS and in the first intron of MIR31HG in human T_{FH} cells²⁰. These results highlighted the important role of miR-31 as intermediate player of

BCL6 gene regulation in human T_{FH} cells. Further studies should be performed to better define BCL6-miR-31 interaction and to clarify mechanisms of miR-31 regulation in human GC T_{FH} cells that might involve other transcription factors (i.e. MAF) or epigenetic modifications (i.e promoter methylation).

In conclusion, our results showed the fundamental role of miRNAs, particularly miR-31, in regulating human T_{FH} cells biology and function, working as molecular bridge between BCL6 and its targets: our data showed that BCL6 represses miR-31 in human T_{FH} cells, leading to positive regulation of its target genes. The posttranscriptional control of gene expression by miRNAs provides a molecular rheostat to modulate and fine-tuning specific genetic circuits and thus is fundamental in the control of T_{FH} cells differentiation and proliferation. This makes miRNAs good candidates for miR-based therapeutic development that could ameliorate autoimmune diseases associated with autoantibody production or could be used in vaccination improvement strategies. Future research studies should investigate the role of other miRNAs belonging to the human T_{FH} cells miRNA-signature we identified, and elucidate how these miRNAs can regulate molecular pathways controlling human T_{FH} cells biology.

Materials and methods

Purification of human lymphocyte subsets from peripheral blood and secondary lymphoid organs.

Buffy coats were obtained from healthy donors at the Istituto di Ricovero e Cura a Carattere Scientifico Policlinico Cà Granda, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll centrifugation. Lymphocyte subsets were purified by sorting on a FACS Aria flow cytometer (BD) by various combinations of surface markers (Table S1).

Fresh human adenoids and tonsils were obtained from pediatric donors at the Istituto di Ricovero e Cura a Carattere Scientifico Policlinico Cà Granda. Tonsils were smashed manually or homogenized using GentleMACS (Miltenyi) to make a single cell suspension. Mononuclear cells were isolated by Ficoll centrifugation and lymphocyte subsets were purified by sorting on a FACS Aria (BD) by various combinations of surface markers (Table S1). The ethical committee of Istituto di Ricovero e Cura a Carattere Scientifico Policlinico Cà Granda approved the use of mononuclear cells from donors for research purposes, and informed consent was obtained from all subjects. The antibodies used for cell sorting were: PE-Cy7 CD4 (clone SK3, BD Pharmingen), VioGreen CD4 (clone VIT4, Miltenyi Biotec), PE CD197 (clone 150503, BD Pharmingen), FITC CD45RA (clone HI100, BD Pharmingen), APC CD45RO (clone UCHL1, BD Pharmingen), PE-Cy5 CD183 (clone 1C6/CXCR3, BD Pharmingen), PE CCR6 (clone 11A9, BD Pharmingen), FITC CD161 (DX12, BD Pharmingen), PE CD127 (clone HIL-7R-M21, BD Pharmingen), FITC CD25 (clone M-A251, BD Pharmingen), PE

CD294 (clone BM16, BD Pharmingen), PE-Cy5 CD3 (clone UCHT1, BD Pharmingen), PE-Cy5 CD56 (clone B159, BD Pharmingen), V450 CD27 (M-T271, BD Horizon), APC-Cy7 CD185 (clone J252D4, Biolegend), PE-Cy7 CD278 (clone C398.4A, Biolegend), FITC CD38 (clone IB6, Miltenyi Biotec).

If not specified otherwise, sorted human CD4⁺ naive and T_{FH} cells were cultured in RPMI 1640 (Euroclone) + 10% of Fetal Bovine Serum (FBS, Gibco) + 1% of penicillin/streptomycin 100x solution (Euroclone).

Purification of mouse lymphocyte subsets from spleens. Five C57BL/6 mice were infused with OT-II thymocytes and pulsed with alum added of ovalbumin (OVA) 100 µg; after 7 days mice were sacrificed and spleens were taken. Splenocytes were pooled together and enriched for CD4⁺ T cells using magnetic beads (Miltenyi) and both CD4⁺ naive T cells and GC T_{FH} cells were sorted accordingly to phenotypical markers (see table S2). After sorting cells were lysed and used later for RNA extraction with mirVana miRNA Isolation Kit (Ambion) and quantification of mmu-miR-31 expression levels using Taqman MicroRNA assay (Applied Biosystems). The experimental procedure was repeated in order to have three independent replicates. All mice were housed in specific pathogen free conditions and treated according to protocols approved by the Animal Care and Use Committee of San Raphael Foundation (Institutional Animal Care and Use Committee).

RNA isolation. Total RNA was isolated using mirVana miRNA Isolation Kit (Ambion) following the standard protocol. Briefly, the lysates were extracted once with Acid-Phenol Chloroform and further purified to yield total RNA with specific miRNA retention. Extracted RNA was quantified with RiboGreen Quantitation Kit (Molecular Probes) on an Infinite F200 plate reader (Tecan Trading AG). All extracted RNA samples were quality controlled for integrity with 2100 Bioanalyzer (Agilent Technologies) and samples with RIN lower than 8 were discarded.

Profiling of miRNA expression. The standard Megaplex protocol (Applied Biosystems) was performed starting from 10 ng of total RNA for each sample with pre-amplification. Total RNA was reversed transcribed using the Megaplex RT stem-loop primers in a 7.5 µl reaction volume through the protocol's default 40 cycles run; 2.5 µl of each RT product were preamplified in a 25 µl reaction volume with Megaplex PreAmp primers to increase detection sensitivity according to manufacturer specifications. Both A and B pools were used in all steps, enabling specific cDNA synthesis of 664 human miRNAs (mirBase v.10.1) and 3 small RNA controls common to all plates (RNA44, RNA48, MammU6). 9 µl of 4-fold diluted pre-amplified RT product were 100 fold diluted in the PCR reaction mix and amplified using TaqMan Low Density Arrays (TLDA) on a 7900HT (Applied Biosystems) according to the standard protocol.

RNA sequencing. Libraries for Illumina sequencing were constructed from 100 ng of total RNA with the Illumina TruSeq RNA Sample Preparation Kit v2 (Set A). The libraries generated were loaded on to the cBot automated clonal amplification system (Illumina) for clustering on a HiSeq Flow Cell v3. The libraries clustered on a HiSeq Flow Cell v3 were then sequenced with a HiScanSQ optical imaging system (Illumina). A paired-end run (with a read length of 101 bases) was performed with an SBS Kit v3 DNA sequencing kit (Illumina). Real-time analysis and base calling was performed with HiSeq Control Software Version 1.5 (Illumina). RNA-seq data representative of human CD4⁺ naive T cell and T_{FH} cell populations from SLO were collected for transcriptome reconstruction. Six biological replicates were analyzed for both populations. The whole data set was aligned to human genome assembly GRCh37 (Genome Reference Consortium Human Build 37) with TopHat software (version 1.4.1)³⁹ for a total of over 1.7x10⁹ mapped paired-end reads (30 million reads per sample on average). These data were also mapped with the aligner STAR (version 2.2.0)⁴⁰. RNA-seq data representative of 13 lymphocyte populations had been performed previously by our group and mapped according to the same criteria⁴¹.

Data treatment, statistics and bioinformatics. The median of three endogenous controls was used as the computed control and the change in cycling threshold (ΔC_T) was calculated. Paired t-test, two-tailed $p < 0.001$ was used for analysis of miRNA expressed in CD4⁺ naive T cells versus GC T_{FH} cells of the same donor. Statistical test and hierarchical clustering were performed on MeV software version 4.5.

TargetScanHuman software, release 6.2, was used for prediction of miR-31 targets; expressed predicted targets were selected among genes expressed in naive and/or GC T_{FH} cells from SLO analyzed by RNA-seq. We chose those miR-31 expressed predicted targets that are at least 2 fold expressed in GC T_{FH} cells in comparison with CD4⁺ naive T cells and whose expression was anti-correlated with the expression of miR-31 (Anti-correlated expressed predicted targets, ACEPTs). We found 347 miR-31 ACEPTs in GC T_{FH} cells that were analyzed using Cuffdiff software and 137 of them resulted significantly more expressed in GC T_{FH} cells in comparison with naive T cells from SLO (t test paired, adjusted p value 0.05).

TaqMan microRNA and Gene Expression assays. For assessment of hsa-miR-31 levels (and for other human miRNAs of the T_{FH} signature) on specific human and mouse lymphocyte subsets single Taqman MicroRNA Assays were used (Applied Biosystems). Five nano-grams of total RNA were used for reverse transcription with specific primers for miR-31 or other miRNAs and U6 snRNA (as normalizer) and Taqman MicroRNA Reverse Transcription Kit followed by real time PCR reaction on a 7900HT Real Time PCR System according to the manufacturer's standard protocol. For assessment of BTLA, SH2D1A, CD40L and 18S (as normalizer) gene expression levels in LVV miR-31 or LVV scramble-transduced T_{FH} cells, TaqMan Gene Expression Assays (Applied Biosystems) were used. Samples were collected 24 hrs post transduction and 50 to 100 nanograms of total RNA were used for reverse transcription with

VILO Reverse Transcriptase (Invitrogen). Diluted cDNA was then used as input for RT-qPCR.

MIR31HG promoter Prediction and BCL6 binding site prediction. The MIR31HG promoter prediction was performed by Promoter Inspector tool of Genomatix suite. 5000 bp upstream the transcription start site of MIR31HG gene. ENSG00000171889, reference annotation Ensembl v. 67, genome GRCh37 was selected for the analysis. Default parameters were considered and the output correspond to the sequence region in which all the requirements are satisfied.

In the same region considered for the previous analysis, the prediction on BCL6 binding site was made. MatInspector tool of Genomatix suite, in which the prediction is based on large library of matrix descriptions for transcription factor binding sites to locate matches in DNA sequences, was adopted with default parameters. 15 binding sites were predicted in the region upstream MIR31HG (Table S3) regardless the sense of transcription. Yellow highlighted binding site couples in Table S3 refer to predicted binding sites that are almost the same (prediction on both DNA strands) and in this work they're considered as one binding site and named as: BS 1 and BS 2 = BS #1/2; BS 4 and BS 5 = BS #4/5; BS 7 and BS 8 = BS #7/8.

ChIP-seq Data. ChIP-sequencing data used in Supplementary Figure 4 are: for CD4⁺ naive T cells, GEO Accession number GSE17312 (Human Reference Epigenome Mapping Project), for GC T_{FH} cells: GEO Accession number GSE59933²⁰.

Molecular biology. The 3' UTRs of five human hsa-miR-31 target genes were PCR amplified (see Primers List 1 below) from cDNA of human testes or human fetal brain and were transferred to the psiCHECK-2 vector (Promega). The 3'UTR sequences used were taken by GenBank (NCBI): BTLA mRNA sequence NM_181780, CD40L mRNA sequence NM_000074, SH2D1A mRNA sequence NM_002351, CXCR5 mRNA sequence NM_001716, CD28 mRNA sequence NM_006139.

Different fragments of promoter region spanning 2.5 kilobases upstream of MIR31HG gene TSS (ENSG00000171889, reference annotation Ensembl v. 67, genome GRCh37) were PCR amplified (see Primers List 2 below) and cloned up-stream of luciferase gene in pGL4.10[luc2] vector (Promega), to form MIR31HG promoter-luciferase reporter constructs, using NheI and XhoI restriction sites. Fragment I is 415 bp, fragment II is 630 bp and fragment III is 2209 bp up-stream of MIR31HG gene first exon. Fragment I contains only minimal promoter, while fragment II and fragment III contain respectively one or three hsa-miR-31 predicted binding sites (Genomatix software). The pGL4.74[*hRluc*/TK] vector (Promega) was used as control vector.

Backbone plasmid for Lentiviral vectors encoding precursor hsa-miR-31 or BCL6 wild type or BCL6 deleted forms were from System Biosciences (SBI): pCDH vector contains a CMV promoter, a multiple cloning site and an EF1 α promoter controlling copGFP expression (SBI, #CD511B). This vector was engineered by the substitution of the portion EF1 α -copGFP with EF1 α controlling the non-signaling version of the human nerve growth factor receptor gene

(Δ NGFR), using NotI and SalI restriction sites. This new pCDH-CMV-MCS-EF1- Δ NGFR was called LVV MOCK Δ NGFR vector.

Hsa-miR-31 lentivirus (LVV-miR-31) was constructed by cloning the stem loop sequence of hsa-miR-31 (miRBASE, MI0000089), together with 200 bp upstream and 200 bp downstream of stem loop sequence, into the MCS of LVV MOCK Δ NGFR vector using SalI and NotI restriction sites. Primer sequences used were *miR-31 F* 5'-CACAAACCTCCTGTGCCTAAC-3' and *miR-31 R* 5'-TGAGGCCCAAATGATGAGAG-3'. A scramble sequence, derived from point mutation in the seed sequence of hsa-125b, was cloned into LVV MOCK Δ NGFR vector using SalI and NotI restriction sites (LVV scramble). The scramble sequence encompassed 200 bp upstream and 200 bp downstream the stem loop sequence of hsa-miR-125b-mutated and it doesn't contain any possible seed sequence.

For BCL6-lentivirus (LVV BCL6) production, human BCL6 coding sequence (CDS) (Ensemble ENST000004068) was PCR amplified and cloned into pCDH MOCK Δ NGFR vector using NheI and NotI restriction sites.

For the expression of BCL6 and its deleted forms under the cumate inducible promoter (CuO), we PCR amplified BCL6 CDS, BCL6 deleted of N-terminal protein-protein interaction domain Broad-Complex, Tramtrack and Bric a brac (BCL6 Δ BTB) or BCL6 deleted of the C-terminal C₂H₂-type zinc finger (ZF) DNA binding domain (BCL6 Δ ZF) into SparQ Dual Promoter Lentivector pCDH CMV5-CuO-MCS-EF1 α -RFP (SBI, #QM512B) using NheI and NotI restriction sites (see Primer List 3). Lentiviral particles were produced according to a standard protocol (System Biosciences User Manual)

and LVVs were called LVV CuO-BCL6-EF1 α -RFP, LVV CuO-BCL6 Δ BTB -EF1 α -RFP and LVV CuO-BCL6 Δ ZF -EF1 α -RFP respectively.

Cumate inducible system for BCL6 expression in HEK293T cells.

In order to stably express CymR repressor in HEK293T cells we transduced these cells with LVV pCDH-EF1-CymR-T2A-Puro (SBI, #QM200VA) at MOI 20. Cells were cultured and selected for ten days in DMEM + 10% Fetal Bovine Serum + 1% penicillin/streptomycin + 1% glutamine + 3 μ g/ml puromycin dihydrochloride (sc-108071, Santa Cruz Biotechnology) and selected also by PCR amplification of the CymR repressor using primers *CymR F* 5'-ATGACATCCACTTTGCCTTTC-3' and *CymR R* 5'-AAGACTTCCTCTGCCCTCTC-3'. Positive clones were cultured always in puromycin enriched medium and the new cell line stably expressing CymR repressor was called HEKCymR. These cells were co-transduced with LVV CuO-BCL6-EF1 α -RFP, LVV CuO-BCL6 Δ BTB-EF1 α -RFP or LVV CuO-BCL6 Δ ZF-EF1 α -RFP, cultured for 10 days in puromycin selection medium and single-cell sorted as RFP^{high} cells, using FACS Aria flow cytometer (BD). Several clones were expanded *in vitro* and tested for their induced ability to express BCL6 proportionally to Cumate added to the medium. Best clones were selected analyzing BCL6 expression at both mRNA (TaqMan Gene Expression Assays, Applied Biosystems) and protein level by flow cytometry (APC-BCL6 antibody, clone 7D1, Biolegend) and used for dual luciferase assay. These clones are able to stably and proportionally express BCL6 and its deleted forms only in the

presence of Cumate in the medium and were called HEKCymR CuO-BCL6, HEKCymR CuO-BCL6 Δ BTB and HEKCymR CuO-BCL6 Δ ZF respectively.

Dual-luciferase assay. For analysis of 3'UTR regions as targets of hsa-miR-31, HEK293T cells were transfected with 10 ng of each psiCHECK-2 construct along with 20 nM hsa-miR-31 duplex or scrambled control miRNA (Qiagen) with Lipofectamine 2000 (Invitrogen). After 24h, cells were lysed and firefly and renilla luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega). Each entire 3' UTR was cloned in psiCHECK-2 immediately downstream of the gene encoding renilla luciferase. Results are presented as the ratio of renilla luciferase activity to firefly luciferase activity.

For the analysis of MIR31HG promoter regions as target of BCL6, HEKCymR CuO-BCL6, HEKCymR CuO-BCL6 Δ BTB and HEKCymR CuO-BCL6 Δ ZF cells were transfected with the different MIR31HG promoter fragments-luciferase reporter constructs (described in Molecular Biology) along with pGL4.74[*hRluc*/TK] control vector (Promega) at a ratio 1:30. All transfections were performed with Lipofectamine 2000 (Invitrogen) and at the same time of transfection increasing amounts (0, 3, 5, 10 and 30 μ g/ml) of Cumate (SBI) were added to the medium in order to express increasing amounts of BCL6. After 24h cells were lysed and firefly and renilla luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega). Results are presented as the ratio of firefly luciferase activity to renilla luciferase activity.

Lentivirus production and transduction. Lentiviral particles were produced according to a standard protocol (System Biosciences User Manual).

CD4⁺ GC T_{FH} cells from human adenoids/tonsils were transduced with either control lentiviral vector or lentiviral vector encoding precursor hsa-miR-31 at a multiplicity of infection of 1x10⁸ transducing units per ml in the presence of polybrene (8 mg/ml) and cytokines IL-21 and IL-6 100 µg/ml. Cells were collected after 24h, and transduction efficiency was assessed by flow cytometry as the frequency of cells positive for DNGFR marker, using APC-CD271 antibody (clone ME20.4-1.H4, Miltenyi Biotec). miR-31 direct targets were analyzed by FACS after surface staining using the following fluorescence-labeled antibodies: VioBlue-CD154 (clone 5C8, Miltenyi Biotec), APC-CD272 (clone MIH26, Biolegend), PE-SAP (clone XLP-1D12, EBioscience). A FACS CantoII and FlowJo887 software were used for data acquisition and analysis. Total RNA was isolated with a mirVana miRNA Isolation Kit (Ambion) and the expression of direct targets of hsa-miR-31 was assessed by TaqMan Gene Expression Assay (Applied Biosystems).

CD4⁺ naive T cells from human adenoids/tonsils were activated for 48h with anti-CD3/CD28 dynabeads (Gibco) and sequently transduced with either control lentiviral vector or lentiviral vector encoding BCL6 (LVV BCL6) at a multiplicity of infection of 1x10⁸ transducing units per ml in the presence of IL-7 2 ng/ml. Cells were collected 5 days post infection and transduction efficiency was assessed by flow cytometry; DNGFR positive cells were sorted by FACS Aria (BD), using APC-CD271 antibody (clone ME20.4-1.H4,

Miltenyi Biotec). miR-31 direct targets were analyzed by FACS after surface staining using the following fluorescence-labeled antibodies: PE-CD278 (ICOS) (clone C398.4A, Biolegend), PE-SAP (clone XLP-1D12, EBioscience). Total RNA of DNGFR positive cells was isolated with a mirVana miRNA Isolation Kit (Ambion) and the expression of hsa-miR-31 was assessed by Taqman MicroRNA Assays (Applied Biosystems).

CD4⁺ T and B cells co-culture: B helper assay. GC T_{FH} cells from SLO were transduced for 24h with either control lentiviral vector (LVV scramble) or lentiviral vector encoding precursor hsa-miR-31 (LVV miR-31). Transduced T_{FH} cells were cultured for 7 days with syngenic B memory cells at a ratio 3:1, in presence of staphylococcal enterotoxin B (SEB 500 ng/ml, Sigma) in RPMI 1640 medium (Euroclone) + 5% low IgG serum (Hyclone Fetal bovine serum U.S super low IgG, GE Healthcare) + 1% penicillin/streptomycin 100x solution (Euroclone). Memory B cells were also cultured alone as negative control, while untreated freshly isolated T_{FH} cells were used as positive control. Immunoglobulins concentrations (IgG/IgM) in cell culture supernatants were measured at day 7 by ELISA.

ELISA. ELISA plates (Maxisorp, Nunc) were coated with capture antibodies (mouse anti-human IgG, clone G18-145, 555784 BD or goat anti-human IgM, I2386 Sigma) for 12h at +4°C, dissolved in PBS 1X (tablets, Sigma), 50 ml/well, at a concentration of 1 mg/ml. After coating, ELISA plates were washed three times with washing buffer composed by PBS1X + Tween 20 0.05% (Sigma) and once

with PBS1X. Wells were then blocked with 100 ml/well of PBS1X + bovine serum albumin 10% (BSA, Sigma) for 2h at room temperature (RT). After blocking, ELISA plates were washed as above and incubated with 50 ml/well of samples or dilutions and standard curves, all diluted in the medium used for B helper assay, for at least 1h at room temperature. Standard curves were made starting from a concentration of 20 ng/ml and then performing serially dilutions 1:2 (IgG from human serum, I4506 Sigma; IgM from human serum, I8260 Sigma). Plates were then washed as above and incubated for 1h at RT with 50 ml/well of detection antibodies (biotin mouse anti-human IgG, clone G18-145, 555785 BD or biotin goat anti-human IgM, B1265 Sigma) diluted in PBS1X + BSA 5%. After this incubation plates were washed again as above and incubated for 1h at RT with 70 ml/well of Streptavidin-poly HRP (Pierce), diluted in PBS1X + BSA 5%. After the incubation with Streptavidin plates were washed four times with PBS1X + Tween 20 0.05% and once with PBS1X and incubated with 50 ml/well of 3,3',5,5' Tetramethylbenzidine (TMB, Sigma). The substrated incubation was done for 2-3 minutes, then stopped by addition of 50 ml/well of H₂SO₄ 1N (Fluka). The absorption was read at 490 nm, with an ELISA reader. The samples were expressed as triplicates or more and concentration of immunoglobulins was calculated by interpolation with standard curve (linear regression).

Primer List 1: Primers used for 3'UTR cloning

Primer name	Target Gene	Type	Sequence
CD40L Fw	CD40L	PCR forward	CACCACGTCCTTTGGCTTAC
CD40L Rev		PCR reverse	ACAATGGAGACTGCAGGTAG
BTLA Fw	BTLA	PCR forward	CACCGTGAGGAGTTAAGTCTGTTTC
BTLA Rev		PCR reverse	CCTTCTCATTGAGCACAATCAC
SH2D1A Fw	SH2D1A	PCR forward	CACCTCCTGATGTCTGCCTGAAAGC
SH2D1A Rev		PCR reverse	GCTCTTGGAAGTGAACATGCAAAC
CXCR5 Fw	CXCR5	PCR forward	CACCTGAAGTTCCGCAGTGAC
CXCR5 Rev		PCR reverse	TCTCTGTGCTGCCTGTACTG
CD28 Fw	CD28	PCR forward	CACCTGACACGGACGCCTATC
CD28 Rev		PCR reverse	AGGCAAGCCAGGTCATC

Primer List 2: primers used for MIR31HG promoter cloning.

Primer Name	Promoter Fragment	Type	Sequence
MIR31HG F1	Fragment I	PCR Forward	AGGTGGACTCCCTCTCCC TTAG
MIR31HG F2	Fragment II	PCR Forward	TCGAAGGACACGGAGGGT TGAG
MIR31HG F3	Fragment III	PCR Forward	GCTTGCTTGCCTCAGTTGA AGTC
MIR31HG R1	All	PCR Reverse	AGCTGCTCACCAAGCTCC TC

Primer List 3: primers used for BCL6 CDS deletions and cloning.

Primer Name	Target Gene	Type	Sequence
BCL6 CDS Fw	BCL6	PCR Forward	ATGGCCTCGCCGGCTGAC
BCL6 CDS Rev		PCR Reverse	TCAGCAGGCTTTGGGGAG
BCL6 DBTB Fw	BCL6	PCR Forward	AACATCATGGCTGTGATGG
BCL6 DZF Rev	BCL6	PCR Reverse	CCCGTTCTCACAGCTAG

Figure and Table Legends

Figure 1: miR-31 is specifically down-regulated in human GC T_{FH} cells. (a) Sorting strategy for human CD4⁺ naive T cells and GC T_{FH} cells isolated from secondary lymphoid organs (SLO: adenoids) of pediatric donors (average age: 7 years). Phenotype and purity of naive and GC T_{FH} cells were checked after sorting (see Table S1). (b) Heat map of the z-scores of signature miRNAs of naive CD4⁺ naive and GC T_{FH} cells sorted as described above. Paired *t* test ($p < 0.001$), two-tailed, HCl with Pearson correlation was used for the statistical analysis using Tmev software. (c) Quantitative RT-PCR analysis of miR-31 expression in CD4⁺ naive T cells and GC T_{FH} cells purified from human SLO of an independent set of pediatric donors (adenoids, empty squares and tonsils, black circles). Data are expressed as mean and s.e.m. * $p < 0.05$ (paired *t*-test, two tailed), n=13. AU, arbitrary units.

(d) Quantitative RT-PCR analysis of miR-31 expression in CD4⁺ naive T cells, T_{H1} and GC T_{FH} cells purified from human secondary lymphoid organs (adenoids and tonsils, black bars), and in CD4⁺ naive T cells, CD45RO⁺CXCR5⁺, CD4⁺ T_{H1}, T_{H2}, T_{H17} and T_{reg} sorted from human peripheral blood (grey bars). Phenotype and purity of naive and GC T_{FH} cells were checked after sorting (see Table S1). AU, arbitrary units, data are expressed as mean and s.e.m. *** $p < 0.001$, GC T_{FH} cells compared to the other CD4⁺ T cell subsets (*t*-test, two tailed). For each subset, n is indicated in Table 1.

Figure 2: CD40L, BTLA and SAP are miR-31 targets. (a) Transcriptome analysis performed by RNA sequencing on human CD4⁺ naive T cells and GC T_{FH} cells from secondary lymphoid organs (SLO: adenoids) of the same donors, used for miRNAs profiling (Fig. 1b). Gene expression values are represented in log₂ FPKM. Transcripts with a fold change between GC T_{FH} and CD4⁺ naive T cells major than 1.5 were considered (blue dots). Red dots are miR-31 anticorrelated expressed predicted targets of (ACEPTs). BTLA, B and T cells attenuator; SAP, SLAM associated protein; CXCR5, C-X-C chemokine receptor 5; CD40L, CD40 ligand are ACEPTs relevant for GC T_{FH} cells biology **(b)** Dual-luciferase assay of HEK-293T cells transfected with luciferase constructs containing 3'UTRs of selected miR-31 ACEPTs, together with synthetic mature miR-31 (mimic miR-31, black bars) or a synthetic control miRNA with scrambled sequence (mimic ctrl, white bars). *SH2D1A* is the gene encoding for SAP. Data are expressed as mean and s.e.m. *** $p < 0.001$, relative to scrambled control (paired *t*-test), n=5. **(c)** Schematic representation of GC T_{FH} cells transduction: cells were transduced for 24h with lentiviral vector encoding the precursor of miR31 (LVV miR-31) or scramble control lentivirus (LVV scramble), without stimulation and in the presence of IL-6 and IL-21 cytokines (100 µg/ml). After 24h GC T_{FH} cells treated with LVV scramble (GC T_{FH} scramble) or LVV miR-31 (GC T_{FH} miR-31) were collected and divided for RNA extraction and cytofluorimetric staining to evaluate miR-31 targets expression. **(d)** Quantitative RT-PCR analysis of *CD40L*, *BTLA* or *SH2D1A* expression in purified GC T_{FH} transduced with LVV scramble (white bars) or LVV miR-31 (black bars). AU: arbitrary

unit, data are expressed as mean and s.e.m, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (paired t -test), $n=3$. **(e, upper graphs)** Quantification of the cytofluorimetric analysis of surface (BTLA) and intracellular staining (CD40L and SAP) in purified GC T_{FH} transduced with LVV scramble (white bars) or LVV miR-31 (black bars). MFI: mean fluorescence intensity. Data are expressed as mean and s.e.m.. * $p < 0.05$, *** $p < 0.001$, relative to scrambled control (paired t -test), $n=3$. **(e-bottom graphs)** Representative histograms of cytofluorimetric analysis of miR-31 targets (CD40L, BTLA and SAP) in GC T_{FH} cells transduced with LVV scramble (grey tinted histograms) or LVV miR-31 (black line).

Figure 3: miR-31 transduced GC T_{FH} cells are less efficient in providing help to B cells. **(a)** GC T_{FH} cells from human tonsils were transduced after sorting with LVV miR-31 or LVV scrambled control for 24h and then put in co-culture with memory B cells (CD3⁺CD56⁻CD27⁺) from the same donor. 30.000 GC T_{FH} cells and 10.000 memory B cells were co-cultured for 7 days in medium with low IgG serum and in the presence of staphylococcal enterotoxin B (SEB) 500 ng/ul. The supernatants were collected at the end of co-culture and used for the quantification of IgG and IgM produced by ELISA. **(b)** Measurement of IgG and IgM by ELISA in supernatants of memory B cells co-cultured with syngenic GC T_{FH} cells for 7 days in the presence of SEB. White and blue histograms depict GC T_{FH} cells transduced with LVV scrambled control (GC T_{FH} scramble) or LVV miR-31 (GC T_{FH} miR-31) respectively. Data are expressed as mean and s.e.m. Controls: memory B cells alone (red histograms). UT: untransduced

GC T_{FH} cells (grey histograms). *** $p < 0.001$, comparison between GC T_{FH} scramble and GC T_{FH} miR-31 (paired t -test), $n=3$.

Figure 4: BCL6 down-regulates miR-31 expression in human tonsillar CD4⁺ naive T cells. (a) Model for BCL6-mediated regulation of GC T_{FH} cells transcripts through modulation of miR-31 expression; (b) Schematic representation of BCL6 overexpression in CD4⁺ naive T cells from SLO: CD4⁺ naive T cells were sorted from human tonsils, stimulated with anti-CD3/CD28 beads, and then transduced on day 2 with LVV encoding BCL6 coding sequence (LVV BCL6) or LVV empty control (LVV MOCK). On day 5 post transduction, transduced cells (called CD4⁺ naive MOCK and CD4⁺ naive BCL6) were sorted as Δ NGFR⁺ (marker of transduction) and RNA was extracted for miR-31 quantification or cells were stained to measure expression of GC T_{FH} specific markers. (c upper graphs). Quantification of the cytofluorimetric analysis of GC T_{FH} cells specific markers after overexpression of BCL6 in CD4⁺ naive T cells from SLO. CXCR5, ICOS and SAP protein expression are shown in purified CD4⁺ naive transduced with LVV MOCK (white bars) or LVV BCL6 (black bars). * $p < 0.05$ (paired t -test). RFI: relative fluorescence intensity. Data are expressed as mean and s.e.m., and are representative of three independent experiments with similar results. (c lower graph) Quantitative RT-PCR analysis of miR-31 expression in naive CD4⁺ naive transduced with LVV MOCK or LVV BCL6. * $p < 0.05$ (paired t -test). AU: arbitrary unit. Data are expressed as mean and s.e.m., $n=3$.

Figure 5: BCL6 binds a specific MIR31HG promoter region in humans. **(a)** Genomic organization of the lncRNA LOC554202 (MIR31HG) locus. Pri-miR-31 is contained in the first intron. Different fragments of a region of about 2500 bp upstream the TSS of MIR31HG gene containing predicted BCL6 binding sites (BS) (red squares), was amplified by PCR (see table S3). Blue square: minimal promoter. **(b)** Schematic representation of the three MIR31HG promoter fragments cloned upstream of Firefly Luciferase in pGL4.10 vector for luciferase experiments. Fragment I is 615 bp long and contains no BCL6 BS; fragment II is 630 bp long and contains one BCL6 BS; fragment III is 2209 bp long and contains five BCL6 BS. **(c)** HEKCymR CuO_BCL6 WT (red bars), HEKCymR CuO_BCL6 Δ BTB (white bars) and HEKCymR CuO_BCL6 Δ ZF (grey bars) clones, stably expressing the repressor CymR and different forms of BCL6 (WT or deleted), were used for dual luciferase assay for 24h on MIR31HG promoter fragments, performed with increased amounts of Cumate in the medium (0, 1.5, 3, 5, 7.5 10 and 30 μ g/ml), in order to induce increased expression of BCL6 (black triangles). Relative luciferase activity (Firefly/Renilla) was measured normalizing results to 0 μ g/ml Cumate condition. Data are expressed as mean and s.e.m. n=3.

Figure 1

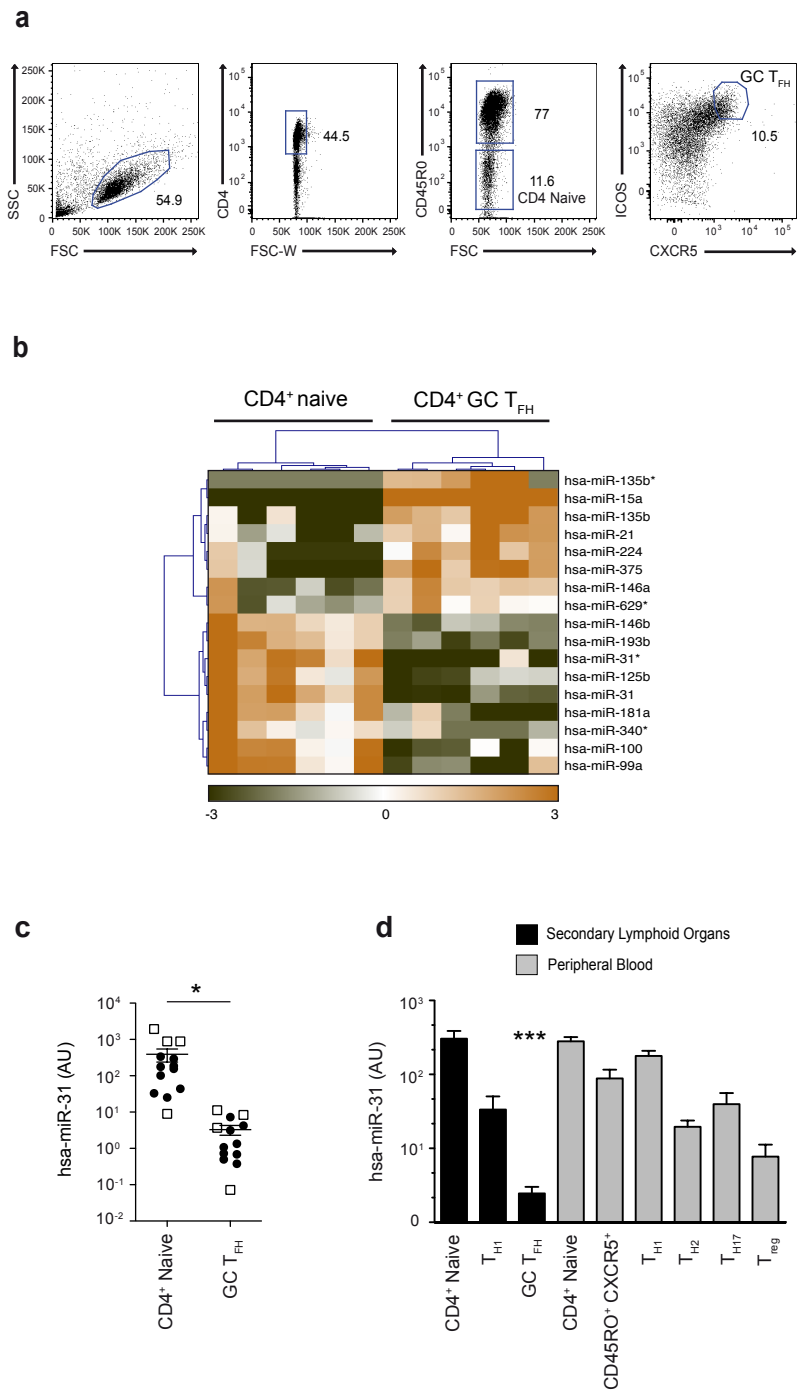
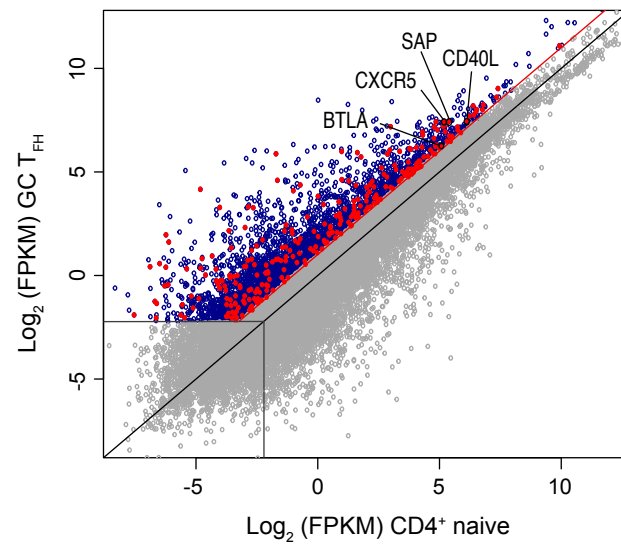
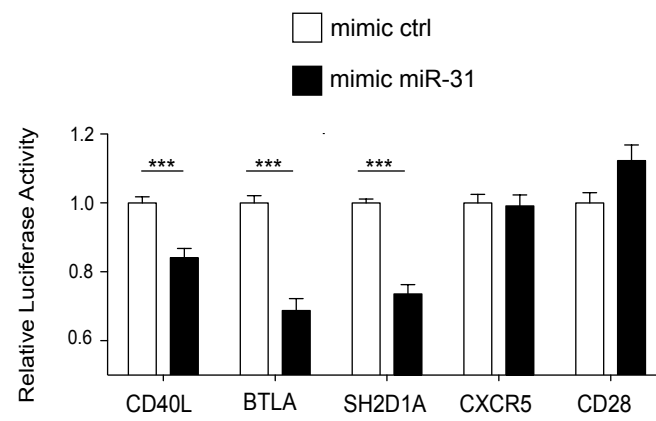


Figure 2

a



b



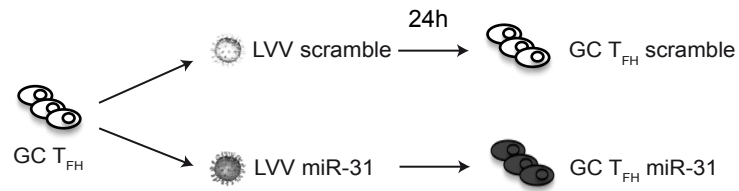
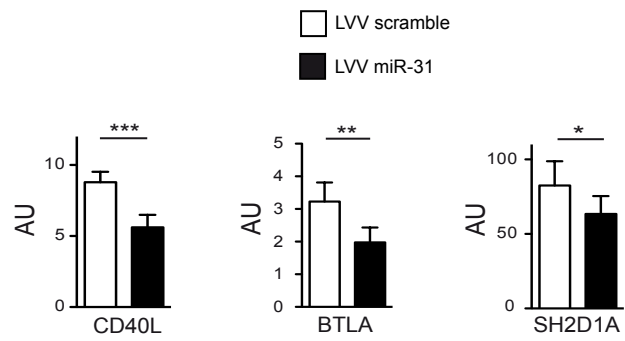
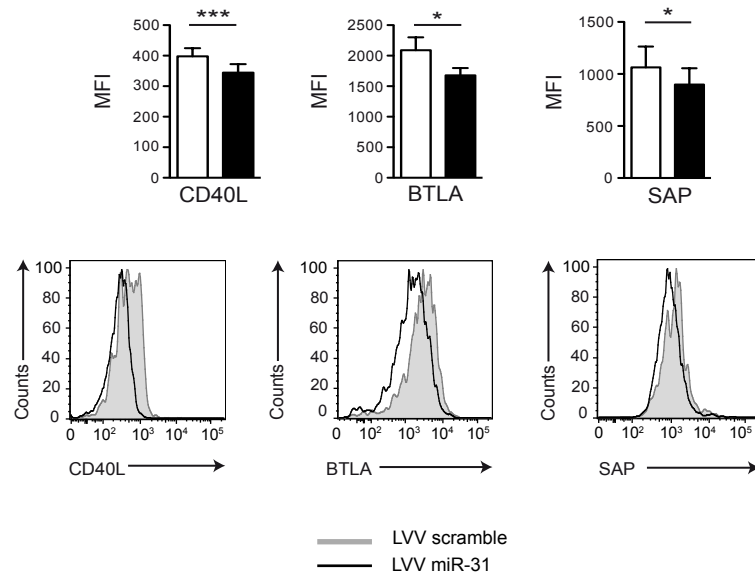
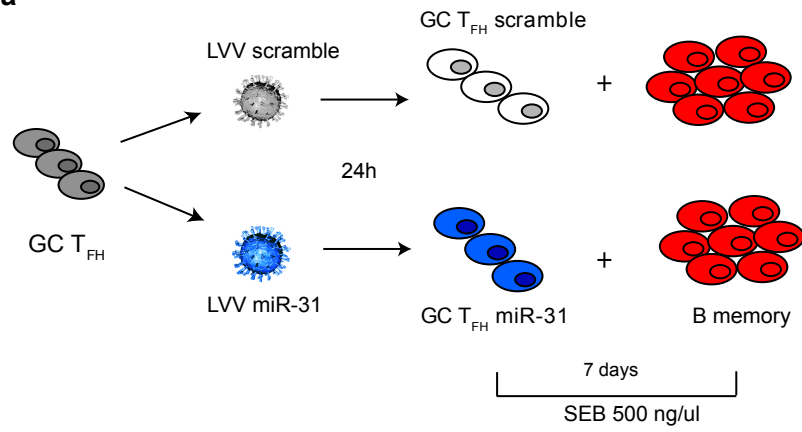
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Figure 3

a



b

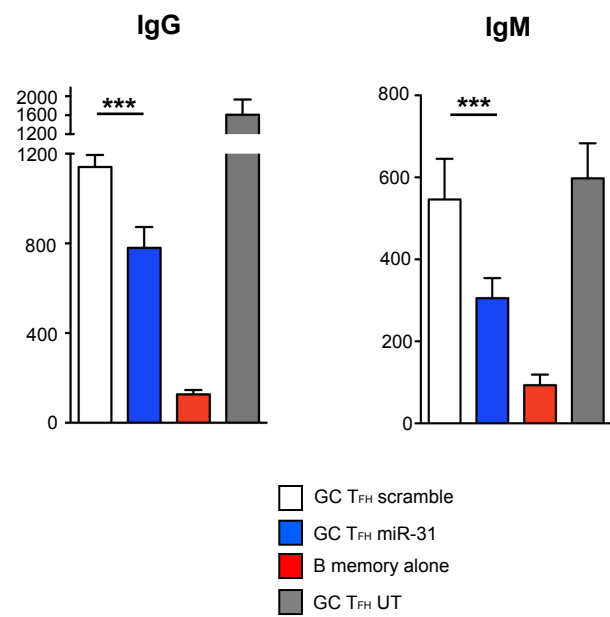
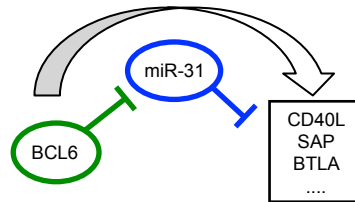
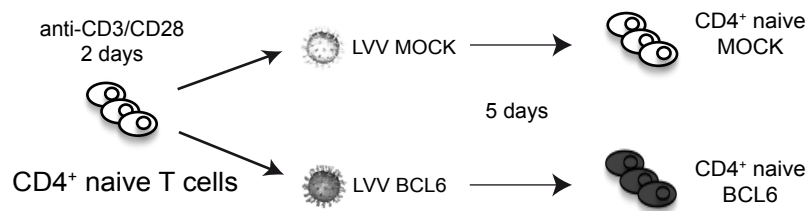


Figure 4

a



b



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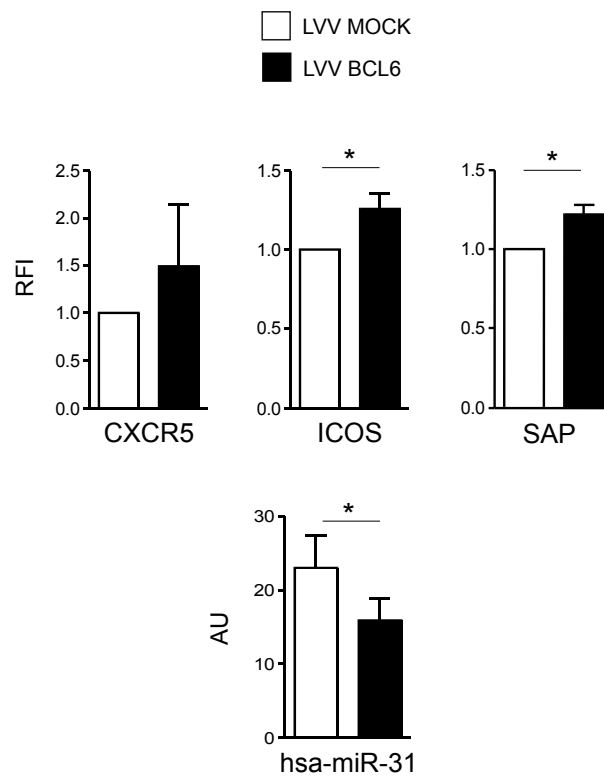
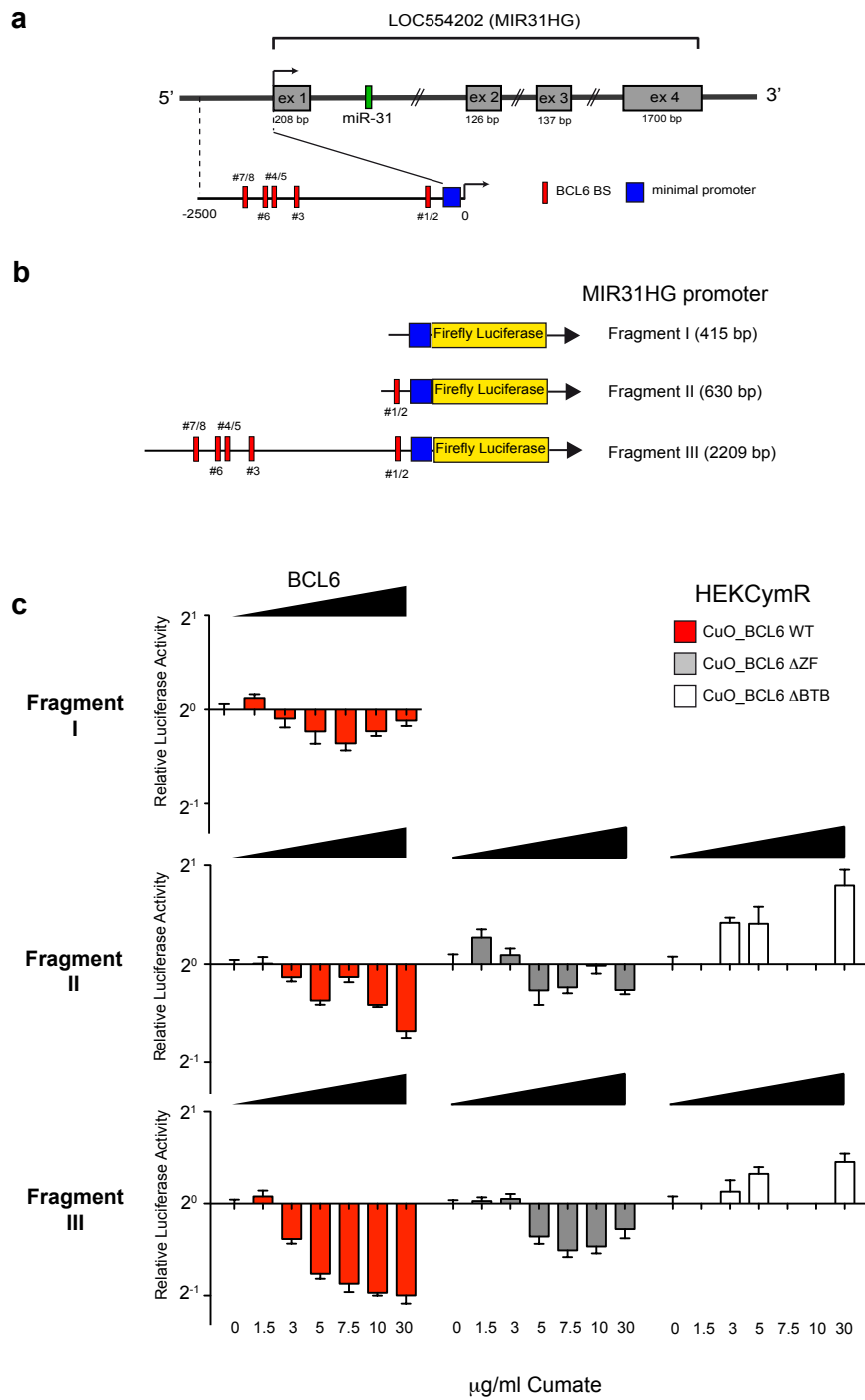


Figure 5



Supplementary Information

Table S1: sorting strategy of human lymphocyte subsets. Sorting of 10 human lymphocyte subsets, isolated from both secondary lymphoid organs (adenoids/tonsils) and peripheral blood (Blood), Subset name, number of donors, purity and markers used for sorting are shown.

Supplementary Figure 1: miR-31 is strongly and specifically downregulated in human T_{FH} cells. Differential expression analyzed by Taqman Low Density Array expressed as $2^{-\Delta\Delta C_t}$ between human CD4⁺ naive T and GC T_{FH} cells (sorted as in table S1) of miRNAs upregulated (light grey histograms, left) or downregulated (dark grey histograms, right) in GC T_{FH} cells in comparison with CD4⁺ naive T cells from secondary lymphoid organs. (*t*-test paired, $p \leq 0.01$). hsa-miR-31 (blue histogram) showed the highest fold change between the two cell subsets. Data are represented as mean and s.e.m., n=6.

Table S2: sorting strategy of mouse lymphocyte subsets. Sorting of CD4⁺ naive and GC T_{FH} mouse lymphocyte subsets, isolated from spleens of OT-II mice 7 days after alum+OVA immunization, by various surface marker combinations (sorting phenotype). Subset name, number of mice per pool, purity and markers used for sorting are shown.

Supplementary Figure 2: mmu-miR-31 is up-regulated in mouse T_{FH} cells. Quantitative RT-PCR analysis of the expression of mmu-miR-31-5p in CD4⁺ naive T cells and T_{FH} cells purified from OT-II

mouse spleens after 7 days of i.v. immunization with alum+OVA 100 µg. Data are presented in arbitrary units (AU) and are expressed as mean and s.e.m. n=3.

Table S3: miR-31 ACEPTs in human T_{FH} versus CD4⁺ naive T cells. List of some of anti-correlated expressed predicted targets (ACEPTs) of miR-31 in human T_{FH} cells compared with paired CD4⁺ naive T cells from SLO, quantified by RNA-seq. Third column represents expression values of miR-31 targets in T_{FH} cells while the fourth column represents expression values of miR-31 targets in CD4⁺ naive T cells. Only targets that have a fold increase of at least 1.5 fold in T_{FH} cells versus paired CD4⁺ naive T cells are shown (Ratio T_{FH}/N, expressed as ratio between values in the third and the fourth column). Genes are listed in table as decreased fold increase between human T_{FH} cells and paired CD4⁺ naive T cells. Bold transcripts highlight genes that have an important role in T_{FH} cells biology and that have been screened in dual luciferase assay as putative miR-31 targets (CXCR5, SH2D1A, BTLA, CD40L, CD28). Data are expressed as mean FPKM, n=6. T_{FH}: follicular helper T cells, N: CD4⁺ naive T cells.

Supplementary Figure 3: Cumate inducible system for BCL6 expression in HEK293T cell lines. (a) Schematic representation of cumate inducible system: the bacterial repressor, CymR (red circle), can bind to the operator sequence (CuO, green box) placed downstream of CMV5, a strong viral promoter that is active in mammalian cells. Once bound, CymR blocks transcription of the

transgene (grey box) from the CMV5 promoter (gene expression OFF). CymR bound to cumate (blue box) is unable to bind to CuO and transcription from CMV5 can proceed unhindered (gene expression ON). **(b)** Schematic representation of LVV encoding different BCL6 constructs, BCL6 WT or deleted of the two main domains, Δ BTB (Broad-Complex, Tramtrack and Bric a brac) or Δ ZF (6 zinc fingers domains), under the cumate-inducible promoter (CuO, green box). **(c)** Flow cytometric analysis of cumate-induced expression of BCL6 WT in the HEKCymR CuO_BCL6 clone use for Dual Luciferase Assay after 24h of transfection. Cumate concentration added directly into the cell medium is shown above the graphs (μ g/ml).

Table S4: BCL6 binding sites (BS) prediction in 5000 bp upstream region of MIR31HG TSS. Region of interest: GRCh37:9: 21559669: 21564668:-1. Promoter Inspector tool of Genomatix suite was used. 5000 bp upstream the transcription start site of MIR31HG gene. ENSG00000171889, reference annotation Ensembl v. 67, genome GRCh37 was selected.

Supplementary figure 4. Top: Chip-seq data of different histonic modifications (H3K4me1, H3K4me3 and H3K27ac) in CD4⁺ naive T and GC T_{FH} cells in MIR31HG locus. **Bottom:** BCL6 ChIP-seq data and BCL6 BS prediction focused on MIR31HG exon 1 region.

Table S1: Purification of human lymphocyte subsets

Subset	Donors (n)	Tissue	Purity (%)	Sorting Phenotype
CD4 ⁺ naive	3	Blood	99.8	CD4 ⁺ CCR7 ⁺ CD45RA ⁺ CD45RO ⁻
CD4 ⁺ T _{H1}	3	Blood	99.9	CD4 ⁺ CD45RO ⁺ CXCR3 ⁺ CCR6 ⁻ CD161 ⁻
CD4 ⁺ T _{H2}	3	Blood	99.1	CD4 ⁺ CD45RO ⁺ CRTH2 ⁺ CXCR3 ⁺
CD4 ⁺ T _{H17}	3	Blood	99.7	CD4 ⁺ CD45RO ⁺ CXCR3 ⁺ CCR6 ⁺ CD161 ⁺
CD4 ⁺ T _{reg}	3	Blood	99	CD4 ⁺ CD45RO ⁺ CD127 ^{lo} CD25 ⁺
CD4 ⁺ CXCR5 ⁺	3	Blood	99.8	CD4 ⁺ CD45RO ⁺ CXCR5 ⁺
CD4 ⁺ naive	6	Adenoids/Tonsils	99.7	CD4 ⁺ CD45RO ⁻ CXCR5 ⁺ ICOS ⁻
CD4 ⁺ GC T _{FH}	6	Adenoids/Tonsils	99.4	CD4 ⁺ CD45RO ⁺ CXCR5 ^{high} ICOS ^{high}
CD4 ⁺ T _{H1}	3	Adenoids/Tonsils	99	CD4 ⁺ CD45RO ⁺ CXCR3 ⁺ CCR6 ⁻ CD161 ⁻
Memory B	3	Adenoids/Tonsils	98.7	CD3 ⁺ CD56 ⁻ CD27 ⁺

Supplementary Figure 1

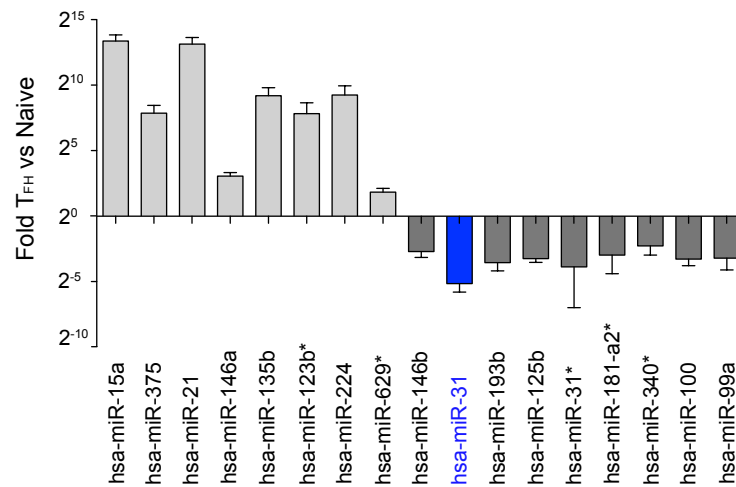
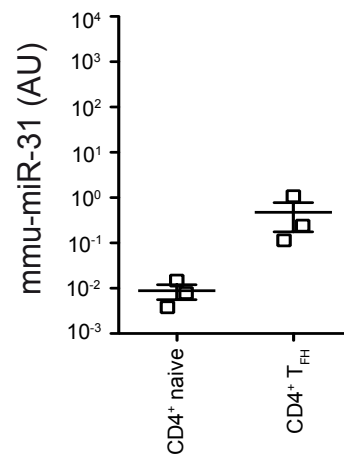


Table S2: purification of mouse lymphocyte subsets

Subset	Pools (n)	Tissue	Purity (%)	Mice per pool	Sorting Phenotype
CD4 ⁺ naive	3	Spleen	99.7	5	TCRβ ⁺ CD4 ⁺ CD19 ⁻ CD44 ^{low} CD62L ^{hi}
CD4 ⁺ T _{FH}	3	Spleen	99.4	5	TCRβ ⁺ CD4 ⁺ CD19 ⁻ CD44 ^{hi} PD-1 ^{hi} CXCR5 ^{hi}

Supplementary Figure 2



Supplementary Figure 3

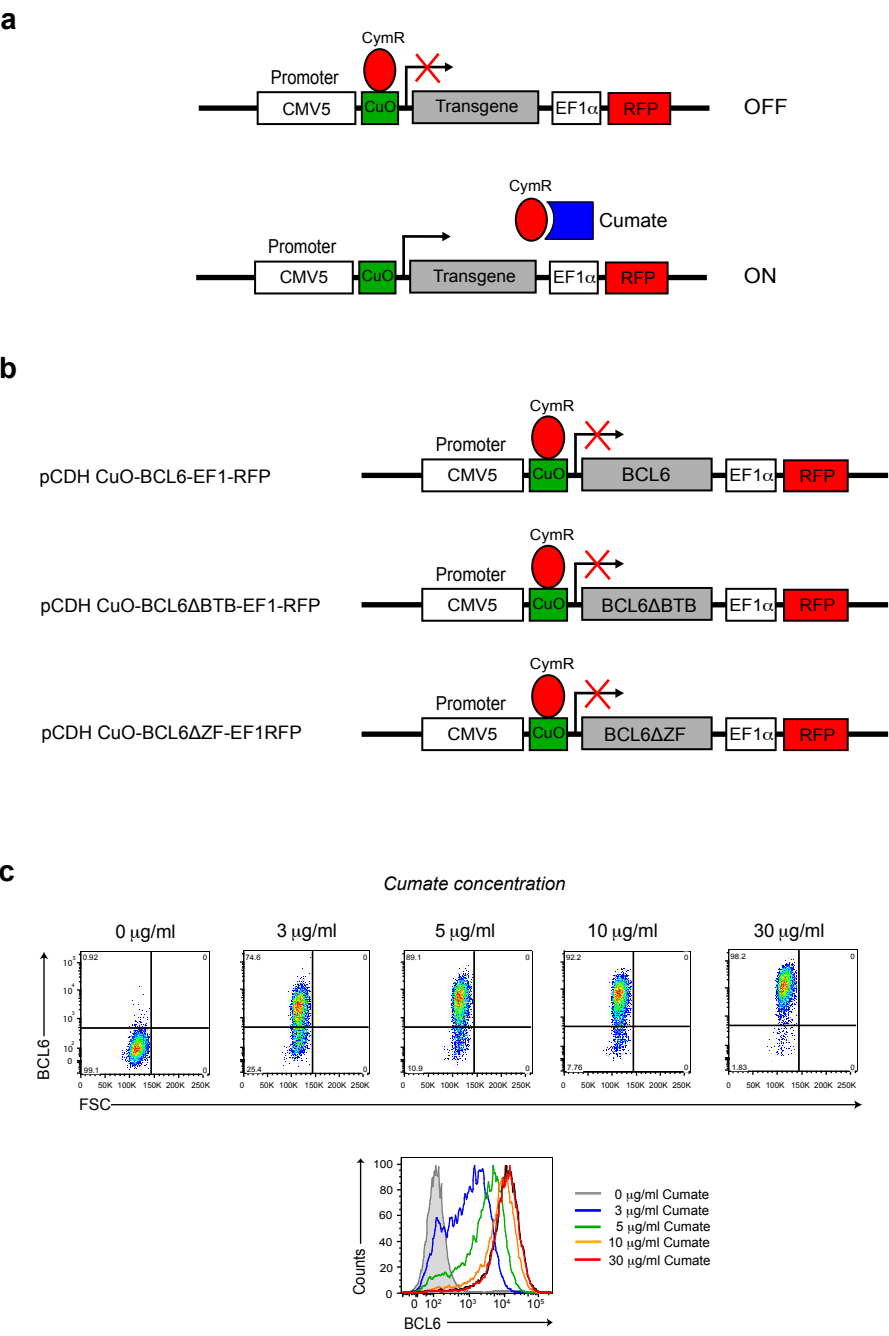


Table S3: miR-31 ACEPTs in human T_{FH} versus CD4⁺ naive T cells

Target gene	Gene name	T _{FH}	CD4 ⁺ Naive	Ratio T _{FH} /N
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	11.001	0.00000020	54729353.234
KSR2	kinase suppressor of ras 2	8.970	0.055	162.345
PTPN14	protein tyrosine phosphatase, non-receptor type 14	13.646	0.215	63.608
APBB2	amyloid beta (A4) precursor protein-binding, family B, member 2	2.415	0.079	30.735
CPM	carboxypeptidase M	48.017	2.052	23.405
RORC	RAR-related orphan receptor C	2.491	0.132	18.839
RGS4	regulator of G-protein signaling 4	0.265	0.014	18.427
SEPT3	septin 3	0.818	0.048	16.892
COL5A1	collagen, type V, alpha 1	0.762	0.051	14.907
CXCR5	chemokine (C-X-C motif) receptor 5	93.580	10.168	9.203
RAB27A	RAB27A, member RAS oncogene family	65.951	7.700	8.566
KRT80	keratin 80	0.317	0.038	8.413
FZD3	frizzled family receptor 3	9.696	1.418	6.838
RAPGEF5	Rap guanine nucleotide exchange factor (GEF) 5	0.798	0.133	5.995
MYO5A	myosin VA (heavy chain 12, myoxin)	36.198	6.053	5.980
SH2D1A	SH2 domain containing 1A	179.409	30.346	5.912
PLXNA2	plexin A2	0.635	0.118	5.405
PHACTR2	phosphatase and actin regulator 2	49.518	9.177	5.396
ADCY6	adenylate cyclase 6	0.313	0.058	5.347
MAFG	v-maf musculoaponeurotic fibrosarcoma oncogene homolog G (avian)	14.582	2.804	5.200
SPECC1	sperm antigen with calponin homology and coiled-coil domains 1	8.413	1.757	4.789
DMD	dystrophin	0.788	0.178	4.426
DHCR24	24-dehydrocholesterol reductase	8.325	1.908	4.364
FAM53B	family with sequence similarity 53, member B	47.260	10.870	4.348
SATB2	SATB homeobox 2	0.391	0.102	3.820
BTLA	B and T lymphocyte associated	37.083	10.079	3.679
TMX4	thioredoxin-related transmembrane protein 4	23.803	7.044	3.379
SNTB2	syntrophin, beta 2 (dystrophin-associated protein A1, 59kDa, basic component 2)	12.590	3.872	3.251
ZDHHC21	zinc finger, DHHC-type containing 21	7.542	2.420	3.116
SLC6A6	solute carrier family 6 (neurotransmitter transporter, taurine), member 6	31.102	10.157	3.062
OSBP2	oxysterol binding protein 2	2.806	0.948	2.959
ZC3H12C	zinc finger CCCH-type containing 12C	0.497	0.172	2.887
SRGAP3	SLIT-ROBO Rho GTPase activating protein 3	5.805	2.090	2.777
JAZF1	JAZF zinc finger 1	24.349	8.858	2.749
RASA1	RAS p21 protein activator (GTPase activating protein) 1	30.436	11.519	2.642
LPP	LIM domain containing preferred translocation partner in lipoma	29.869	11.462	2.606
ZFP36L1	zinc finger protein 36, C3H type-like 1	198.872	76.555	2.598
PPP2R5C	protein phosphatase 2, regulatory subunit B', gamma	288.026	113.699	2.533
IFFO2	intermediate filament family orphan 2	30.028	11.977	2.507
VAV3	vav 3 guanine nucleotide exchange factor	10.287	4.354	2.362

GCLM	glutamate-cysteine ligase, modifier subunit	3.972	1.705	2.329
AMOTL1	angiomotin like 1	0.273	0.120	2.282
SUFU	suppressor of fused homolog (Drosophila)	8.250	3.628	2.274
CD40LG	CD40 ligand	47.774	21.599	2.212
MBOAT2	membrane bound O-acyltransferase domain containing 2	4.037	1.865	2.164
GNA13	guanine nucleotide binding protein (G protein), alpha 13	52.099	24.119	2.160
NDRG3	NDRG family member 3	20.785	9.634	2.158
XKR6	XK, Kell blood group complex subunit-related family, member 6	4.782	2.327	2.055
SERTAD2	SERTA domain containing 2	34.151	16.710	2.044
TFRC	transferrin receptor (p90, CD71)	96.957	47.694	2.033
WIPF1	WAS/WASL interacting protein family, member 1	289.884	143.069	2.026
SPOPL	speckle-type POZ protein-like	9.260	4.627	2.001
RNF183	ring finger protein 183	0.251	0.132	1.907
STAM2	signal transducing adaptor molecule (SH3 domain and ITAM motif) 2	10.575	5.555	1.904
CD28	CD28 molecule	142.171	75.913	1.873
UBE2K	ubiquitin-conjugating enzyme E2K	37.241	20.226	1.841
C15orf57	chromosome 15 open reading frame 57	7.409	4.168	1.778
ATP8A1	ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1	43.323	24.393	1.776
CAMK2D	calcium/calmodulin-dependent protein kinase II delta	17.429	9.913	1.758
RAB5B	RAB5B, member RAS oncogene family	29.701	16.945	1.753
MFAP3	microfibrillar-associated protein 3	8.161	4.692	1.739
DPY19L3	dpy-19-like 3 (C. elegans)	5.405	3.110	1.738
NPR3	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	0.236	0.136	1.732
VEZT	vezatin, adherens junctions transmembrane protein	25.925	15.094	1.718
TNS1	tensin 1	0.389	0.228	1.703
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	51.799	31.595	1.639
LBH	limb bud and heart development homolog (mouse)	228.873	140.669	1.627
AGRN	agrin	8.198	5.070	1.617
MAP3K14	mitogen-activated protein kinase kinase kinase 14	20.444	12.701	1.610
SLC30A6	solute carrier family 30 (zinc transporter), member 6	9.058	5.634	1.608
PC	pyruvate carboxylase	5.827	3.636	1.602
PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	19.257	12.056	1.597
SSH1	slingshot homolog 1 (Drosophila)	10.569	6.629	1.594
PPP2R2A	protein phosphatase 2, regulatory subunit B, alpha	37.693	24.220	1.556
CTIF	CBP80/20-dependent translation initiation factor	0.464	0.299	1.552
RBBP4	retinoblastoma binding protein 4	29.030	18.772	1.546
OXSRI	oxidative-stress responsive 1	18.209	11.827	1.540

Table S4: chromosomal localization of BCL6 binding sites (BS) on MIR31HG promoter region.

Region of interest: GRCh37:9: 21559669: 21564668:-1.

	START	END	Score
Promoter	21559669	21564668	
BS 1	21560247	21560263	0.819
BS 2	21560248	21560264	0.831
BS 3	21561430	21561446	0.776
BS 4	21561520	21561536	0.893
BS 5	21561522	21561538	0.867
BS 6	21561543	21561559	0.810
BS 7	21561737	21561753	0.885
BS 8	21561739	21561755	0.811
BS 9	21562781	21562797	0.776
BS 10	21562884	21562900	0.782
BS 11	21562954	21562970	0.811
BS 12	21563335	21563351	0.784
BS 13	21563594	21563610	0.809
BS 14	21563756	21563772	0.882
BS 15	21563923	21563939	0.805

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3. Conclusions and perspectives in translational medicine

The two main themes debated in this thesis that could have an impact in translational medicine are microRNAs (miRNAs) and their role in human follicular helper T cells (T_{FH}) biology.

miRNAs are highly conserved small non coding RNAs that regulate gene expression at the post-transcriptional level: they recruit the miRNA-induced silencing complex (miRISC) and guide it on the mRNAs targets, ultimately causing their deadenylation and subsequent degradation^{1, 2}. miRNAs participate in many physiological processes and regulate cellular processes such as differentiation, proliferation and apoptosis³. They are also important in regulating the molecular pathways that control the development and function of innate and adaptive immune responses, and they have been implicated in the control of key aspects of $CD4^+$ T helper cells differentiation, function and maintenance of cellular identity^{4, 5}. Gene expression profiling studies have demonstrated alterations in miRNA expression in a wide range of human diseases and in many cases, functional studies have linked miRNA dysregulation as a causal factor in disease progression⁶. Alterations of miRNAs expression were found in cardiovascular disorders, neurodegenerative diseases, metabolic disorders and several types of cancers⁷. Moreover miRNAs have a direct role in several immunological pathologies, and their expression is modified in many autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS) and inflammatory bowel disease (IBD)⁸.

T_{FH} cells are an independent subset of CD4⁺ T helper cells specialized in providing help to B cells to generate high-affinity plasma cells and memory B cells in GCs of secondary lymphoid organs, having a critical role in the mediation of humoral adaptive immunity. Therefore it is not surprising that alterations in expression of several molecules regulating T_{FH} cells differentiation and function cause a dysregulated behaviour of GC and extrafollicular T_{FH} cells, contributing to several immunodeficiencies or autoimmune disorders, such as SLE, Sjögren's syndrome, juvenile dermaromyositis and autoimmune thyroid diseases⁹ (Table 3). Despite T_{FH} cells number is not altered in most of these autoimmune conditions, the levels of circulating blood memory T_{FH} cells (CD4⁺CXCR5⁺ICOS^{hi} or PD-1^{hi}) are generally increased, and this often correlates with autoantibody titers, frequencies of circulating GC B cells and plasma cells, and with disease severity⁹. Together with increased serum levels of IL-21, these data are indicative of altered T_{FH} cells activity and could be useful biomarkers for predicting disease outcome. Several experiments in mice also showed a direct correlation between the number of T_{FH} cells and the number of GC B cells, of plasma cells and antibody production^{10, 11}. Given the fundamental role of T_{FH} cells in maintaining immunological humoral tolerance and the lack of studies defining miRNAs role in human T_{FH} cells biology, we wanted to characterize human T_{FH} cells miRNOME to identify miRNAs implicated in T_{FH} cells differentiation and function that might represent novel therapeutic targets for the development of miRNA-based treatments of autoimmune diseases. Hsa-miR-31, that we have characterized in this work, could be a promising therapeutic target due to its role in the regulation of SAP,

CD40L and BTLA, molecules critical for the development and function of T_{FH} cells. SAP binds the immunotyrosine switch motifs (ITSMs) in the cytoplasmic tails of SLAM receptors, thus mediates the prolonged T_{FH}-B cells adhesion and promotes IL-4 secretion by T_{FH} cells. CD40L, together with IL-21 and IL-4, is a potent anti-apoptotic and proliferation signal for B cells while BTLA controls functional activity of T_{FH} cells by inhibiting excessive IL-21 secretion by these cells¹²⁻¹⁵. SAP and CD40L are also essential for the T_{FH} cells survival because in the absence of a stable cognate contact with B cells, provided by CD40-CD40L signals, T_{FH} cells undergo apoptosis and are not able to sustain GC reaction any more. Thus, the unique and specific low level of miR-31 expression in human T_{FH} cells among the other CD4⁺ T helper cell subsets and the relevance of miR-31 targets in human T_{FH} cells biology, make it an ideal target to possibly modulate the altered T_{FH}-driven signals provided to B cells during autoimmune disorders or even in T_{FH} cells-related cancers. Specifically miR-31 over-expression in human GC T_{FH} cells could in turn downregulate SAP and CD40L or reduce BTLA-mediated secretion of IL-21, reducing aberrant and excessive helping signals provided by T_{FH} cells also to autoreactive B cells, thus blocking the development of autoimmune pathologies. On the contrary miRNAs that in T_{FH} cells are known to promote their differentiation and proliferation, such as miR-17~92 cluster¹⁶, could be downregulated in order to contrast T_{FH} cells-driven autoimmune diseases. Other microRNAs of the T_{FH} cells-signature we identified should be characterized in order to select other candidates that might be suitable

for a miRNA-based therapy against T_{FH} cells-driven autoimmune diseases.

miR-31 expression is altered in several types of cancers (i.e. breast cancer, colorectal cancer, bladder cancer, melanoma)¹⁷: miR-31 has a fundamental role in regulating the invasion-metastasis cascade and targets genes involved in the regulation of cell proliferation and apoptosis¹⁷. Giving the unique expression profile of miR-31 in T_{FH} cells and its role in the development of several tumors, further investigations must be performed in order to evaluate its involvement molecular pathways of T_{FH} cells-related cancers, and eventually used to develop anti-tumor therapeutic strategies.

Regulation of T_{FH} cells biology and function through modulation of key molecules and/or T_{FH}-specific miRNAs can have a deep impact also in vaccines improvement strategies. Vaccines represent the most cost-effective life-saving device in history, by controlling infectious disease, improving the quality of life and increasing life expectancy^{18, 19}. Vaccination is aimed at inducing a specific immune response that will protect the immunized individual from a pathogen for a long period of time. Each vaccine is unique in terms of its composition and formulation. Vaccines can be classified mainly in three groups: live attenuated vaccines, inactivated vaccines and DNA vaccines. The first group consists of a weakened form of the infectious agent itself that can mimic the kind of protective immunity induced in people who survive live infection. The attenuated form can reproduce but it is unable to elicit the disease in normal, healthy people. Some examples of these vaccines are those against measles, mumps, rubella, polio vaccines and yellow fever. The second group consists of intact heat or

chemically-inactivated bacteria or viruses (often referred to as whole cell vaccines) or extracts of those agents sometimes referred to as acellular, subunit, or fractional vaccines. The components of these vaccines are not able to reproduce, do not cause disease, and are typically given in multiple doses to elicit immune response. The aim of this second kind of vaccines is to stimulate the protective response without triggering the disease. Inactivated vaccines include some of those for influenza (flu), rabies, hepatitis A and B, pertussis and tetanus (that uses the toxin secreted by the bacterium).

DNA vaccines are composed by a gene sequence of interest (generally encoding antigenic or immune adjuvants genes) PCR amplified, inserted into a plasmid backbone and delivered to the inoculation site, generally skin (intradermally), subcutaneum or muscle. Using the host-cellular machinery the plasmid enters the nucleus of local cells (i.e. keratinocytes or myocytes) including APCs, where the expression of plasmid-encoded genes is followed by generation of foreign antigens as proteins or as peptide strings. APCs present vaccine-derived endogenous peptides on MHC I and II molecules to T cells in draining lymph nodes, inducing both cellular and humoral immune responses²⁰. Indeed, they activate and induce the expansion of both CD8⁺ cytotoxic lymphocytes and CD4⁺ T cells that in turn induce B cell activation and antibody secretion²⁰. These kind of vaccines were developed mostly against influenza virus but despite their several advantages, they have low immunogenicity and a lower efficiency in large animals and humans and must be provided in multiple doses or using adjuvants.

The ability of a vaccine to create a long-lasting protective immunity is sometimes enhanced when it is combined with an adjuvant, a substance that attracts additional inflammatory cells to the site of injury and stimulates them to release more and different cytokines. Few adjuvants have been licenced for use clinically around the world and these includes alum (aluminum salt-based adjuvant), AS04 (composed by a TLR4 ligand adsorbed to alum) and oil-in-water emulsions (MF59, AS03)¹⁸; several studies aimed at improving vaccines efficacy, mainly investigated how to do this through modulation of adjuvant composition.

In general, some vaccines are only partially effective even after multiple vaccinations (e.g., acellular pertussis)¹⁹ and development of better vaccination strategies that require fewer boosters is an important goal, especially in developing countries. Improving vaccines-induced long-lasting immune responses is challenging. The goal of T-cell based vaccines is to stimulate innate immune cells (DCs or macrophages) at inducing antigen-specific memory T cells differentiation: these memory T cells should persist longer after the antigen has been eliminated and confer protection against subsequent infections. Beside this, for many vaccines it is the quality and the persistence of antibody responses that matters¹⁸. T_{FH} cells play a key role in the induction of a long-lived high-affinity antibody response and further improvement in vaccine strategies should take into account mechanisms that regulate T_{FH} cells differentiation and development. Indeed, the characteristics of the vaccine antigen influences the levels and the duration of immunity¹⁹, depending most of all from T_{FH} -B cells interaction. In broad terms, vaccines can be

categorized into three general classes: multivalent non-protein antigen, monovalent protein antigen, and multivalent protein antigen (fig. 15)¹⁹. In the first group there are polysaccharide vaccines (i.e. Pneumovax-23), that are able to elicit B cell activation through clustering of the B cell receptor (BCR) but without an associated protein in the vaccine, this occurs in the absence of cognate T cell help provided by T_{FH} cells. This T-independent antibody response provides only a limited duration of protection in elderly subjects and antibody responses decline to baseline levels within 3–5 years after vaccination. Moreover, T-independent antibody responses fail to establish memory and re-vaccination with purified polysaccharides and have been found to lower serum antibody responses, and decrease the frequency of antigen-specific memory B cells. Vaccination with monovalent protein antigens (such as tetanus and diphtheria) are less likely to trigger BCR clustering than multivalent antigens but since BCR-mediated antigen uptake results in presentation of peptide antigens to T_{FH} cells, this facilitates B-T cells contact and the additional costimulation from these interactions elicits stronger and more durable antibody responses than that observed with T-independent antigens vaccines. Tetanus and diphtheria toxoid vaccination, belonging to this class, induce intermediate to long-lived antibody responses that decline with an 11-year and 19-year half-life, respectively. Finally multivalent vaccine antigens and many natural acute viral infections elicit long-term immunity after only one or two antigenic exposures, that is due to multiple factors including increased crosslinking of the BCR and enhanced antigen presentation to T_{FH} cells, resulting in longer T-B cell “dwell time” interactions that

provide the signals that may be necessary for imprinting a longer lifespan in daughter cells destined to become long-lived plasma cells. According to this model, measles infection induce long-term protective immunity, often sustained for life. Thus, a multivalent antigen triggers strong B cell activation as well as effective T_{FH} cells help¹⁹. (Fig. 15). Although more studies are needed, the combination of increased BCR clustering and increased antigen presentation to T_{FH} cells by multivalent proteins may play a role in imprinting an increased plasma cell lifespan and sustained antibody production¹⁹. We can speculate that modulation of T_{FH} cells number can improve vaccines mechanisms and lasting of protection, overcoming the composition of vaccine itself: increasing the number of T_{FH} cells and promoting their survival can sustain the survival of high-affinity plasma cells and memory B cells for longer time and promote long lasting high-specific antibody response. Modulation of T_{FH}-specific miRNAs might be an approach to be pursued in the future to improve T_{FH} cells survival and differentiation.

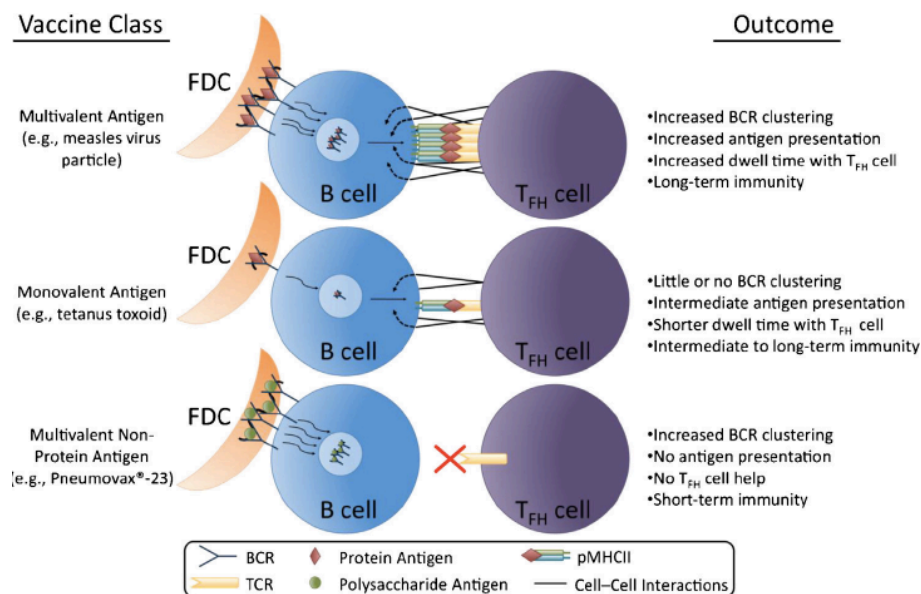


Figure 15: Characteristics of a vaccine antigen determine subsequent levels and duration of immunity¹⁹. A putative multivalent protein antigen is shown in comparison to a monovalent protein or non-protein antigen. In this example, B cell clones extract antigen from follicular dendritic cells (FDC), followed by processing (protein antigens only) and presentation to T follicular helper (T_{FH}) cells. Several lines of evidence indicate that multivalent interactions increase B cell receptor (BCR) clustering and improve the ability of B cells to secure antigen from antigen presenting cells. This multivalent interaction also stabilizes the antigen-BCR complex and leads to an increase in peptide-loaded MHC Class II complexes (pMHCII). Increased presentation of pMHCII to cognate T_{FH} cells, in addition to other cell-to-cell interactions (e.g., CD40-CD40L, etc.), can lengthen the dwell time between B cells and T cells, improving the survival for a particular B cell clone.

During the past decades, several tools have been developed in order to modulate (increase or decrease) miRNAs expression and to study the effect on miRNAs alteration in normal cellular processes and human diseases. These strategies can be exploited also for the development of miRNA-based therapy. In some pathologies miRNAs expression is reduced so miRNA-base therapy must replace the missing miRNAs. The expression of exogenous small RNA in cells is possible by transient or stable transfection or lentiviral vector transduction of several constructs, such as pri-miRNA transgene, pre-miRNA, mature miRNA/miRNA* (miRNA mimic, synthetic dsRNA duplexes that mimic native miRNAs), small interfering RNA (siRNA) or short hairpin RNA (shRNA)²¹ (Fig. 16).

On the contrary, miRNAs that are elevated in diseases can be targeted by antisense oligonucleotide inhibitors (called also antagomirs) that binds to the miRNA by Watson-Crick base pairing, causing miRNA tailing with adenosines or uridines and subsequently degradation, preventing miRNA binding to its target mRNAs. These synthetic antisense oligonucleotides contain 2'-O-methyl-modified ribose sugars, terminal phosphorothioates and at the 3' end a cholesterol group, which helps to deliver the antagomir to cells. More recently locked nucleic acid (LNA) modifications have been developed: in the LNA conformation the ribose ring is connected by a methylene bridge between the 2'-O and 4'-C atoms thus "locking" the ribose ring in the ideal conformation for Watson-Crick binding. This enhances the stability of the duplex and its survival *in vivo*, making it resistant to endonucleases degradation. Another tool to reduce miRNAs expression is the use of lentiviral vectors to deliver miRNAs decoys,

called miRNAs sponges, into cells. miRNA sponges contain complementary binding sites to miRNAs of interest that sequester mature miRNA of interest and reduce its expression²¹ (Fig. 16).

To deliver synthetic constructs such as miRNA mimic or antagomirs into cells without the use of lentiviral vectors, several strategies have been developed to prevent phagocytosis from immune cells, filtering by the kidney and to enhance cellular delivery. The first is the use of liposomes, lipid vesicles made up from lipid bilayers with an aqueous core that contains the nucleic acid cargo and allows the delivery of miRNA mimic or antagomir into the cells: the anionic charge of the nucleic acids and their hydrophilicity is counterbalanced by the cationic lipids, resulting in net positive charge that enables liposomes to bind the anionic cell surface molecules. More recently encapsulation technologies using nanoparticles that can be modified to selectively target specific tissue or cell types using surface antibodies (Abs) were developed for the treatment of immune diseases²². This kind approach holds considerable promise for future miRNA-based studies. Another technique that is still at the initial phase of study, is miRNAs delivery for therapeutic applications using endogenous exosomes, nano-sized vesicles (30-90 nm in size) that are produced by many cell types, including dendritic cells (DC), B cells, T cells and tumor cells. Exosomes have been demonstrated to physiologically contain mRNA and miRNAs and to shuttle them between cells²³, therefore they could be potentially exploited for gene therapy, particularly for the delivery of therapeutic nucleic acids, such as miRNAs (Fig. 17). Exosomes from a patient can be used as non-immunogenic gene delivery vectors for the nucleic acid-based therapy

of the same individual, overcoming some of the safe issues about gene therapy, that must be safe, efficient, organ/cell specific and should not cause immune or inflammatory response.

Recent years have seen considerable progress in the field of miRNA-based therapies²⁴. RNA interference (RNAi) is an efficient process of posttranscriptional gene silencing that has been developed into a new technology in biopharmaceutical fields of science. RNAi products are mainly short interference RNA (siRNA), but also short hairpin RNA (shRNA) and microRNA (miRNA), that can cause inhibition of protein synthesis and regulate important biological processes. These products have been used largely as therapeutic drugs for the treatment of different pathologies, such as different types of cancer or as antiviral therapy²⁵. Despite the large use of siRNAs, a Phase II clinical trial succeeded in using an antagomiR for the treatment of hepatitis C (HCV): *Jannsen et al.*²⁶ showed that the use of Miravirsen, a LNA-modified DNA phosphorothioate antisense oligonucleotide that sequesters mature miR-122, a miRNA that promotes HCV RNA propagation in human hepatocytes, causes a dose-dependent reduction of HCV RNA levels in patients with chronic HCV infection. However, there are serious challenges that need to be overcome before miRNA-targeted therapies can be used in immunological diseases, most of all the development of methods for the delivery of these modalities specifically to T_H cells in patients. With regard to this, a recent study of *Wahlgren et al.*²⁷ showed an efficient delivery of siRNA to T cells and monocytes using plasma exosomes derived from the same patient. These studies open the field for new types of approaches using miRNAs as therapeutic drugs, eliminating the immunogenicity usually

associated to other delivery systems used to shuttle foreign nucleic acids into cells. Further studies should be performed in order to overcome other difficulties associated with miRNA-based therapies, such as miRNAs redundancy (ability of one miRNA to regulate the expression of several targets) and efficiency (how many miRNAs molecules are needed to have an effect without being toxic).

In conclusion, understanding the role of microRNAs in modulating key molecular pathways of human T_{FH} cells biology provides the knowledge that might be exploited for the development of new strategies in facing autoimmune diseases and to improve vaccines mechanisms of action. Indeed, the broader and general field of non-coding RNAs that can regulate T_{FH} cells biology should be investigated in deep in the future, in order to have a specific and clear picture of gene expression modulation in this important T cell subset that is able to regulate humoral immune system and to promote a high-affinity antibody response against several pathologic threats.

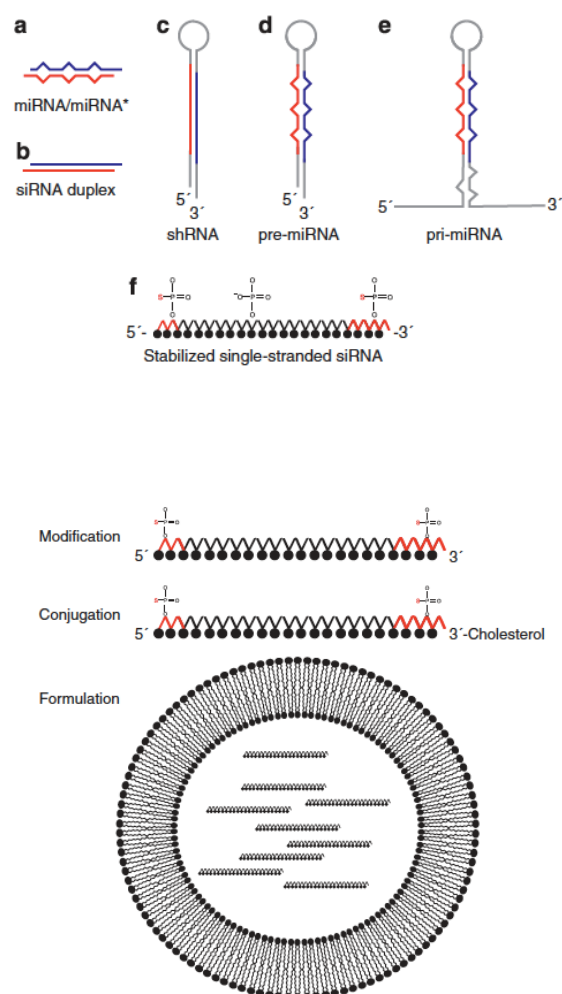


Figure 16: miRNA replacement strategies²¹. (Top) (a) mature miRNA/miRNA* duplex; (b) small interfering RNA duplex; (c) short hairpin RNA (shRNA); (d) premiRNA; (e) pri-miRNA; (f) modified single-stranded RNA. (Bottom) Strategies for delivery of anti-miRNA oligonucleotides to cells *in vivo*. Modification: black filled circles represent 2'-O-methyl, 2'-O-methoxyethyl or 2-fluoro modified nucleotides. Conjugation: antagomirs are 2'-O-methyl oligonucleotides conjugated to cholesterol at their 3' ends, and contain phosphorothioate linkages between nucleotides at both ends in place of natural phosphate linkages. Formulation: lipid nanoparticles are lipid vesicles containing

therapeutic RNA, delivering it to cells and promoting fusion with the phospholipid bilayer of cell membranes.

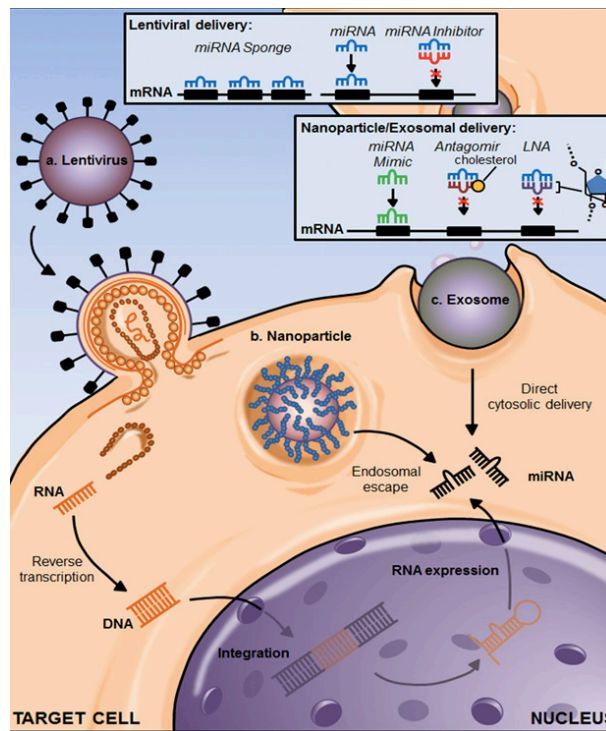


Figure 17: Approaches to target miRNAs²⁸. (a) Lentiviral delivery of expression vectors for miRNA overexpression or miRNA sponges and inhibitors that can block endogenous miRNA function. Synthetic miRNA mimics can be delivered into cells using (b) nanoparticles or (c) exosomes. Nanoparticle and exosomal delivery also can be used for LNAs or antagomirs that bind and inhibit miRNAs.

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